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The Bacteriophages of *Pseudomonas pyocyanea*

2. Bacteriophage Reproduction in an Iridescent Strain

By LOÏS DICKINSON and SHEILA CODD

The Research Department, Bacteriology Division, Boots Pure Drug Co. Ltd., Nottingham

SUMMARY: Two new compounds inhibited the increase of phage when two phages of *Pseudomonas pyocyanea* were grown on an indicator strain. These compounds also inhibited the appearance of iridescence in a lysogenic strain of this organism.

One iridescent lysogenic strain showed a rise in phage titre at 1½–2 hr., which corresponded to the latent period of the phage when it acted on an indicator strain. Both these increases were inhibited by the compounds. This lysogenic strain also showed a rise in phage titre at 4 hr. but this was unaffected by the compounds. The final phage titre of the lysogenic culture was not affected significantly, unlike the complete and permanent suppression of phage increase when free phage acted on an indicator strain. Cultures and filtrates of three lysogenic strains of *Ps. pyocyanea* showed a great increase in phage titre when assay plates (against an indicator strain) were incubated for a further period after the initial reading at 18 hr.

Many workers have described iridescent strains of *Pseudomonas pyocyanea.* Warner (1950) gave a detailed description and concluded that the iridescent clearing was not caused by bacteriophage action but was a distinct lytic phenomenon, probably the result of bacterial variation. Don & van den Ende (1950) studied thirty strains of *Ps. pyocyanea* with particular reference to the metallic lytic effect and bacteriophage action but the relationship, if any, between these two effects remained obscure. Following an investigation of drug action on the phages of *Ps. pyocyanea* (Dickinson, 1948), an explanation was sought for the lack of action of drugs on phages associated with iridescent cultures of this organism. It was decided to investigate the reproduction of phage in one of these cultures. The present paper reports this work and includes observations which correlate iridescence with bacteriophage action in this particular strain.

The following abbreviations and definitions are used in this paper. C1–C10 are cultures of *Ps. pyocyanea*; Pa, Pb, Px are bacteriophages of *Ps. pyocyanea*; F1–F10 are the filtrates of cultures after filtration through Ford's Sterimats (Seitz type). *Free phage* is phage present in an extracellular form. *Lysogenic bacteria* are bacteria which can perpetuate bacteriophage without the intervention of free phage. *Indicator strain,* for example C10, is a strain sensitive to the phage of a lysogenic strain. Only phages acting on C10 were assayed in this work. Phage giving rise to plaques on C10 in an 18 hr. assay is called 18 hr. phage and phage which only gives clear plaques after 48 hr. is termed 48 hr. phage. *Latent period* is the time interval between infection of a culture by bacteriophage and liberation of phage by that culture. The finely adjusted system of Ellis & Delbrück (1939) with only one phage particle/bacterium/tube was unnecessary for this work since the 'burst size' was not required.

EXPERIMENTAL

Strains. The same strains of *Ps. pyocyanea* were used as by Dickinson (1948), particularly the iridescent cultures C1 and C7X and the non-iridescent cultures C7 and C10. Strain C7X was originally obtained from the spleen of a mouse which had been inoculated intraperitoneally with a mixture of C7 and the filtrate of C1. Strain C1 was later found to yield two bacteriophages, named Pa and Pb. C10 was the indicator strain used to assay Pa, Pb and also Px, the phage in filtrates of C7X.

Media. The chemically-defined medium consisted of: lactic acid, 2·4 g.; NaCl, 5 g.; $(NH_4)_2HPO_4$, 1 g.; KH_2PO_4, 1 g.; $MgSO_4.7H_2O$, 0·2 g.; distilled water 1 l., pH 7·0 (Dickinson, 1948). Better growth was obtained when 1 % casein hydrolysate (vitamin free) was added.

Titration of bacteriophage. One ml. volumes of serial dilutions of the preparations in Ringer's solution were added to 0·5 ml. of a 24 hr. nutrient broth culture of C10; 3 ml. melted nutrient agar were added and the mixture 'sloped' and incubated for 18 *hr. only* (this time is particularly important in view of later work). Plaques were then counted.

Investigations into bacteriophage reproduction

*Latent period of phages Pb and Px on C*10. Preparations of Pb or Px containing about 10^6 phage particles/ml. were mixed in broth with a suspension of C10 containing about 10^8 viable bacteria/ml. This solution was diluted so that the final dilution bottle contained 10–20 phage particles/ml. and 1 ml. volumes were then distributed in a series of tubes, which were incubated in a water-bath at 37°. At intervals, duplicate or triplicate sample tubes were removed and assayed for phage (against C10, as above) in the sample tube. When high counts were expected the 1 ml. volumes were diluted before the addition of the indicator culture. A typical result to show the variance encountered on counts, before and after the rise in titre, is shown in Table 1. Using the same technique, it was found that T2 phage of *Escherichia coli*

Table 1. *Latent periods of phage Pb on* Ps. pyocyanea *strain C*10 *in nutrient broth and in Ringer's solution*

	Phage count/tube (triplicate readings)					
Time (min.)	In broth			In Ringer's solution		
0	6	9	12	1	6	3
15	7	13	7	6	9	0
30	3	6	10	18	4	3
45	10	16	5	31	23	19
60	13	8	7	9	11	10
75	7	8	22	6	8	14
90	—	18	28	10	10	7
105	66	82	80	17	8	15
135	86	60	122	14	22	22
180	82	116	109	10	10	17

gave a value for the latent period of 20–25 min., compared to 21 min. quoted by Delbrück (1946).

Culture C7X. A 24 hr. culture of strain C7X was diluted so that the required number of 18 hr. phage particles (i.e. phage particles revealed by the 18 hr. assay described above) were in the sample tubes in 1 ml. volumes. Assays against C10 were made at intervals; the assay tubes therefore contained a small number of C7X host cells.

Bacterial counts were made by the pour-plate method, using nutrient agar; colonies were counted after 2 days' incubation.

Testing of drugs. The methods of Dickinson (1948) were employed when testing compounds for the inhibition of iridescence on nutrient agar plates. Certain compounds were also tested for their effect on the growth of bacteriophage; the methods used were as above, the compounds having been previously incorporated in the final dilution bottle at a concentration known to permit good host growth.

RESULTS

Description of strain C7X of Pseudomonus pyocyanea *and its phage, Px*

There is evidence that *Ps. pyocyanea* strain C7X was produced *in vivo* from C7 and F1 (the filtrate from the iridescent C1 strain containing at least two distinct phages, Pa and Pb). Filtrates from C7 did not act on any of our strains of *Ps. pyocyanea* (Dickinson, 1948). Serologically Px (the phage in filtrates of C7X) appeared very similar to Pa but quite unrelated to Pb in neutralization tests. Thus, rabbit antiserum against Pb phage did not neutralize Pa or Px phages, whereas antisera against Px or Pa phage were equally effective against either of these two phages, but were inactive against Pb phage (Table 2). Plaques of Px and Pa on C10 were very similar in appearance. Neither Px, Pa nor Pb gave rise to an iridescent culture when grown on C10. Unfortunately, C7 was a very slowly growing strain and gradually died out. In these circumstances one can dispute the origin of C7X but it was quite distinct from any of our other strains. It produced a green, but not a blue-green, pigment and all colonies examined gave rise to the metallic sheen; typical colonies are shown in Pl. 1, fig. 1.

Table 2. *Neutralization of Pa, Pb and Px phages by homologous and heterologous antisera.*

	Number of phage particles neutralized by 1 ml. serum		
Sera	Pa	Pb	Px
Anti-Pa	1,000–10,000	<10	1,000
Anti-Pb	<10	1,000,000	<10
Anti-Px	10,000–100,000	<10	10,000–1,000,000
Normal serum	<10	<10	<10

Equal volumes of inactivated sera dilution 1/10 and serial tenfold dilutions of the phage preparations were incubated for 30 min. at 37° and then assayed for phage. Only approximate counts were made, i.e. <10, 10–100 (hence the values in the tables).

Latent periods of phages Pb and Px on strain C10

Phage Pb, originally obtained from C1, had been used as a test virus in chemotherapy work so that much information was available regarding its growth on C10. Table 1 shows that, in nutrient broth, there was a marked rise in phage titre between 1½ and 1¾ hr. There was no increase in phage when the test was carried out in Ringer's solution. In lactate medium, with or without 1% casein hydrolysate, the latent period was also about 1½–2 hr. Phages Pa and Px behaved similarly to Pb; results for Px are given in Fig. 1. Their long latent periods are possibly explained by a slow rate of adsorption since, under the same conditions in broth, the quickly adsorbed T2 phage of *Esch. coli.* gave a latent period of 20–25 min. whereas the slowly adsorbed T1 and T5 phages took much longer than the times quoted by Delbrück (1946). Attempts to find the adsorption time by centrifugation and the use of homologous anti-sera were not successful.

Bacteriophage reproduction in strain C7X

Pa, Pb and Px phages when grown on strain C10 in either nutrient broth or defined medium regularly gave 10^9 to 10^{10} phage particles/ml. However, C7X never yielded phage preparations with as many as 10^8 particles/ml. when counted, filtered or unfiltered, by an 18 hr. assay against C10; the host colony count was of the order of 10^9/ml. Strain C7X was therefore investigated: (i) in the presence of initial 18 hr. phage, and (ii) at a dilution which eliminated 18 hr. phage but not host cells. The results of one experiment are given in Fig. 1 and show that when there was 18 hr. phage initially in the tube, there was a marked rise in phage titre at 1½–2 hr. This period of about 1¾ hr. is particularly interesting because this is the duration of the latent period under similar conditions, of Pa, Pb and Px phages acting on C10.

When there was no initial 18 hr. phage and about 10 host cells/ml., there was no increase of phage at 1½–2 hr., but phage appeared at 4–5 hr. On statistical grounds a single 18 hr. phage particle would be present in approximately 1 tube in 10 so the possibility exists that this small amount was not detected in the assays until after the second cycle or after cell division. However, out of 95 tubes read from 0–4 hr. all were negative or contained 1 or 2 phage particles only, whereas 7/8 read after 4 hr. had titres greater than 25/tube; the negative sample was a 4 hr. one.

Action of various compounds on phage production

A number of compounds have been found to suppress the growth of Pb an Px phages at concentrations not affecting the growth of C10. However, none of these had any effect on the 24 hr. phage titre of unfiltered cultures of strain C7X. Proflavine was first investigated, but the assay tubes were so difficult to read that it was useless for this work. Attention was therefore concentrated on the following two compounds (p. 6):

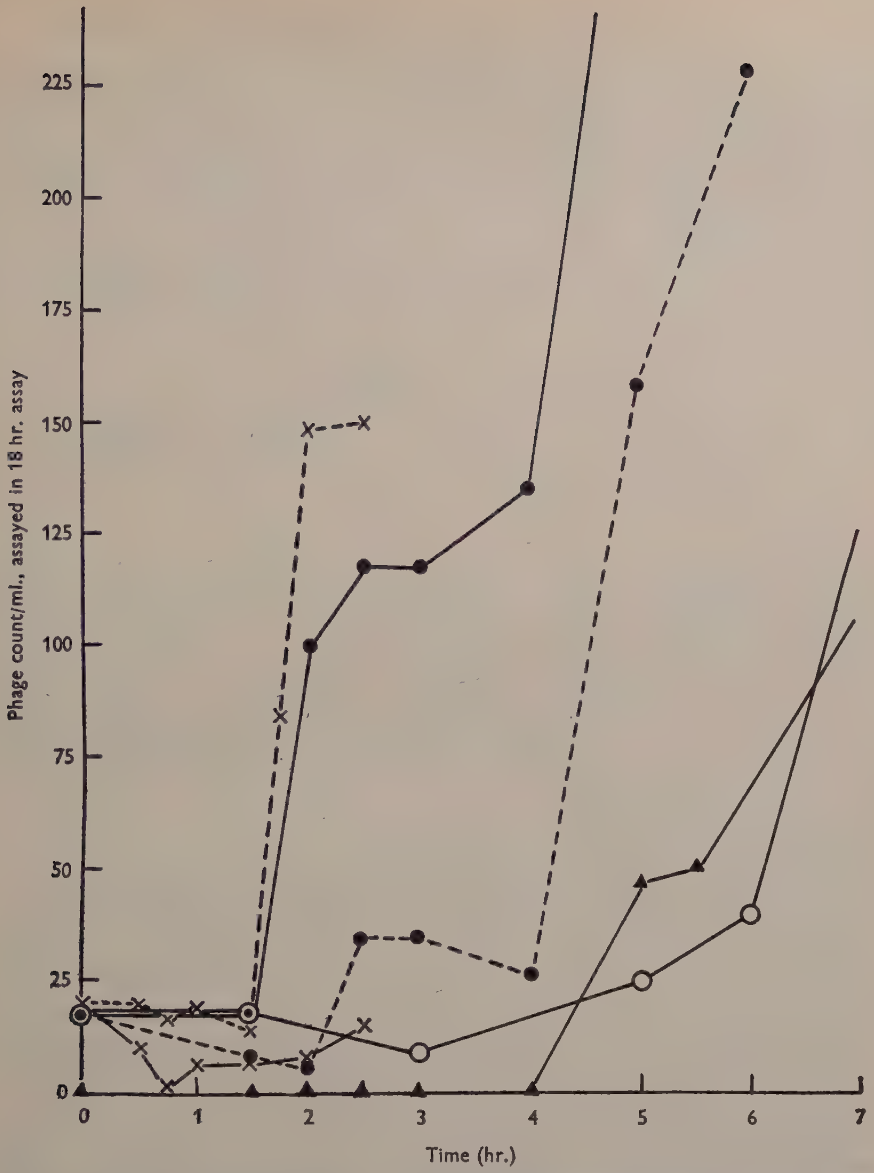
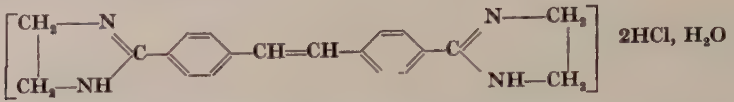


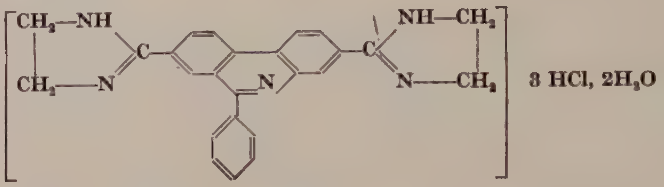
Fig. 1. Production of bacteriophage in cultures of *Ps. pyocyanea* strain C7X and of phage Px on *Ps. pyocyanea* strain C10, with and without compounds A or B.

Curve symbol	Test	Initial 18 hr. phage particles/ml.	Initial bacterial colony count/ml.
×----×	Phage Px on strain C10	20	1000
×——×	Phage Px on C10 (+compound B)	20	1000
●——●	Phage C7X (with initial 18 hr. phage)	17	900
●----●	Phage C7X (with initial 18 hr. phage+compound A)	17	900
○——○	Phage C7X (with initial 18 hr. phage+compound B)	17	900
▲——▲	Phage C7X (no initial 18 hr. phage)	0	9

Compound A—4:4-Bis-(2-dihydroglyoxalinyl) stilbene dihydrochloride, monohydrate (Oxley & Short, 1947):



Compound B—2:7-Bis-(2-dihydroglyoxalinyl)-9-phenyl-phenanthridine trihydrochloride, dihydrate:



Either of these compounds, incorporated in lactate medium or broth, inhibited the increase of phage shown by control cultures of C10 inoculated with phages Pb or Px. Cultures originally containing an inoculum of approximately 20 phage particles/ml. and 10^5 bacteria/ml. normally contain, after 24 hr. incubation, 10^8 phage particles/ml.; one loop of such cultures when 'spotted' on a nutrient agar plate seeded with C10 lyses the area on which it has been spotted. Chemicals can be incorporated in the medium, and when the phage does not multiply an area of 'no lysis' on the spot is observed. Provided that C10 itself has grown in the tube (observed visually) this indicates that the compound is active in some way. Preliminary tests showed that compound A inhibited the increase of phage at 0·1 mg./ml.; it permitted host growth equal to that of a control tube at 1 mg./ml. Compound B inhibited phage growth at 0·01 mg./ml.; it permitted host growth equal to the control at 1 mg./mg. Furthermore, when these drug-treated cultures were incubated for periods up to 7 days there was no increase of phage. After 48 hr., when the bacterial count in all tubes was between 10^8 and 10^9 organisms/ml., phage titres/ml. were as follows: control, 28×10^8/ml.; with compound A (1 mg./ml.), 0/ml.; with compound B (1 mg./ml.), 0/ml.

Growth curves for strain C10, with and without phage in the presence and absence of compounds A and B. In assessing the significance of the action of a compound on virus infections the effect of the compound on the host is obviously important. The action of compounds A and B on the growth rate of culture C10 was therefore investigated in the presence and absence of phage Pb. After the initial addition of phage to host in broth, the solution was diluted and inoculated into 50 ml. volumes of casein hydrolysate medium containing: (*a*) 0·1 mg./ml. of compound A; (*b*) 0·1 mg./ml. compound B; (*c*) control bottle without compound. The initial host colony count was 10^4/ml. and the initial phage count was 120/ml. In a similar test carried out at the same time the effects of the compounds on C10, grown in the absence of phage, were investigated. Colony counts and phage counts were made at intervals by the plate methods. The results showed (Figs. 2, 3) that compounds

A and B at a concentration of 0·1 mg./ml. had no effect on the growth rate of C10. Both compounds inhibited the phage increase when phage Pb was grown on C10, the phage count falling to zero after several hours' incubation. There was sufficient phage left viable, however, to have given an increase at 1½–2 hr., when the control count was rising rapidly. The host colony counts at 12 and 24 hr. were similar whether phage had multiplied or not, although the cultures containing compound A or B looked more turbid (about twice) than the control. *Complete* lysis of C10 has never been observed during these investigations; whether this is due to the growth of phage-resistant strains has not been determined. The unexpected rise in the host colony count between 0 and 3 hr. followed by a fall, which did not occur when the host cell alone was under test, has not been explained.

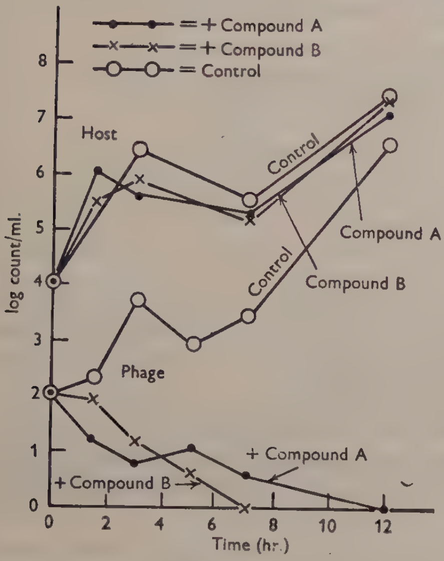


Fig. 2

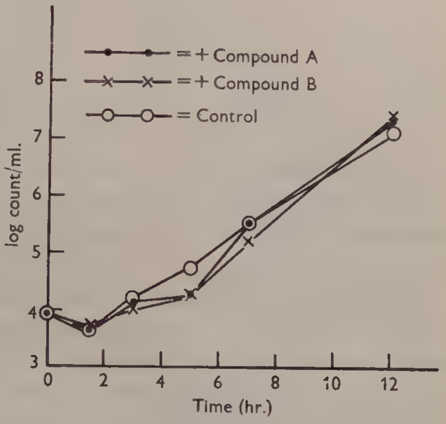


Fig. 3

Fig. 2. Growth curves for phage Pb and host strain C10 of *Ps. pyocyanea* when grown together, with and without compounds A and B (0·1 mg./ml.).

Fig. 3. Growth curve for host strain C10 of *Ps. pyocyanea*, with and without compounds A and B (0·1 mg./ml.).

Contact test of compounds A and B on phage Px. In order to see whether the compounds were simply inactivating free phage, Px phage suspensions containing (*a*) 24×10^4 and (*b*) 24 phage particles/ml. were left in contact with the compounds for 24 hr. at 37° in casein hydrolysate medium.

Results appear in Table 3 and show that the fall in titre was not sufficient to account for the action of the compounds A and B by a contact action alone. The treated phage was in all cases capable of infecting untreated host cells and giving high phage titres. When the initial phage titre in the contact tests was high (*a*) and dilutions were necessary for the phage count, then the compounds were so diluted that they could not be acting by preventing the adsorption of phage on the host cells in the assay, since they were diluted to 1/100th of their effective concentrations. Similar results were obtained with Pb phage. These experiments suggested, but did not prove, that compounds A and B inhibited phage multiplication within the cell. Further work is in hand on this point, but the data reported here enabled the compounds to be used against strain C7X with the knowledge that they prevented phage action without apparent damage to the host cell.

Table 3. *Contact action of compounds A and B on phage Px of* Ps. pyocyanea *at 37° in casein hydrolysate medium*

Medium		Phage titre/ml. after 24 hr.	
Compound	(mg./ml.)	Initial titre = 10^6	Initial titre = 100
Control		48×10^4	50
A	1·0	1×10^2	8
A	0·1	5×10^2	10
B	1·0	77×10^2	3
B	0·1	67×10^2	2

Effect of compounds A and B on the latent period of phages Pb and Px on C10. Compounds A and B at 0·1 mg./ml. prevented the rise in titre at 1½–2 hr in experiments on the effect of these compounds on the latent period. Results for the action of compound B on phage Px appear in Fig. 1.

Effect of compounds A and B on bacteriophage reproduction in strain C7X. Compounds A and B at concentrations which did not affect the growth rate of the host cells had no significant effect on the bacteriophage titres after 24 hr. incubation of strain C7X. In a growth curve experiment using compound B at 0·1 mg./ml. the curves for control and treated cultures ran parallel to each other, both for host cell and 18 hr. phage counts. The compounds (at 0·1 mg./ml.) were also tested in the presence of a low initial amount of 18 hr. phage (17 particles/ml.); the results are shown in Fig. 1. With compound B there was no rise in titre at 1½–2 hr. such as occurred in the control C7X tube; with compound B the rise was very small and also delayed. However, with both compounds there was a marked rise in titre at 4 hr. and the final phage titre approached that of the control. This rise in titre at 4 hr. corresponded to the rise which occurred with C7X cultures when the initial inoculum contained host cells but no 18 hr. phage, as well as to the second rise occurring in tubes which did contain initial 18 hr. phage.

Attempts were made to see whether filtered cultures of strain C7X contained phage after growth in the presence of compounds A and B. Results showed a considerable fall (phage titres were about 100/ml., compared to 10^6/ml. for untreated control filtered cultures). However, compounds A and B also lowered the phage titres in C7X filtrates when added immediately before filtration of the culture so part, if not all, of the fall in titre, could have been due to losses during filtration.

Increase of phage titre by incubating assay tubes for 48 *hr.*

The results of the experiments on the phage reproduction in strain C7X presented several problems. In particular, it was difficult to explain the fact that, when the host cells produced phage at 4–5 hr., the initial phage counts for these tubes should not have given rise to plaques in the assays. As the host cells only appeared to liberate phage after a certain delay, the assay tubes were incubated for a further period after the first reading at 18 hr. The titre, as assayed at 48 hr., was then found to be as much as 100 times that read at 18 hr. This phenomenon was better observed when the assays were made by the agar layer technique where, instead of 'sloping' the tubes, the contents were poured on to a nutrient agar plate. In every plate tested there was an increase (Table 4). These figures showed that the phage counts at 48 hr. were almost as high as the host cell counts made from the same dilution as the phage counts (both counts being made by the plate method). These results explained many puzzling facts, in particular the occasional hazy backgrounds in the assay tubes of C7X *cultures* and the variable appearance of plaques in such assays.

Table 4. *Px phage titres in* 24 *hr. broth cultures assayed at* 18 *and* 48 *hr. against* Ps. pyocyanea *strain C*10

(*a*, *b* and *c* indicate separate tests. 18 and 48 hr. assay carried out on same plate.)

			18 hr. assay	48 hr. assay	Host cell colony (count/ ml.)
Material assayed	Derived from	Log dilution	(Phage plaques/ml.)		
Culture C7X (*a*)	24 hr. broth cultures	−6	34	125	140
(*b*)	24 hr. broth cultures	−6	2	119	816
(*c*)	24 hr. broth cultures	−6	64	156	—
(*c*)	24 hr. broth cultures	−7	0	12	63
(*c*)	24 hr. broth cultures	−8	1	1	9
Filtrate of C7X(=F7X)	24 hr. broth cultures	−5	45	100	0
		−6	3	9	0
Culture (C10+F7X)	24 hr. broth culture of C10+F7X, grown together	−6	336	336	—
		−8	6	6	—
Filtrate (C10+F7X)	24 hr. broth cultures of C10+F7X, grown together	−6	585	590	0
		−8	6	6	0
Cultures (48 hr. F7X (*a*) plaques+C10)	48 hr. plaque from assay of filtrate from C7X	−8	20	20	—
(*b*)		−8	5	5	—
Culture (18 hr. F7X plaque+C10)	18 hr. plaque from assay of filtrate from C7X	−6	189	196	—
		−7	16	16	—

Preliminary studies on the nature of the two types of plaques have indicated that the picture is very complex, but the following experiments have been carried out and confirmed. As stated above, when cultures of C7X were assayed against C10, both 18 and 48 hr. plaques were found. The same phenomenon occurred with strains C1 and with the non-iridescent strain

C1R, which was isolated from C1 in 1948. *Filtrates* of all these cultures also gave both types of plaques. When filtrates of C7X were sown on C10 and the resulting cultures or filtrates assayed, only 18 hr. plaques were produced. The obvious explanations was that the 48 hr. plaques were derived from host cells, but the results with filtrates do not support this theory. Filtrates of the cultures C7X, C1 and C1R were tested for sterility by adding 1 ml. volumes of undiluted filtrates to 9 ml. nutrient broth and incubating for 7 days. No growth of bacteria occurred. Filtrates of these strains have been kept at +5° for 18 months and are still bacteriologically sterile.

It should be noted that in assays of C7X, the 48 hr. plaques first appeared as hazy areas (almost as a background to the sharply defined 18 hr. type plaques), when the assay plates had been incubated for about 24 hr. They gradually became more distinct although, even at 48 hr., they were never as clear as the 18 hr. type.

A 48 hr. plaque from an assay of a filtrate of culture C7X was picked off with accompanying C10 cells, and incubated in broth. The resulting culture was assayed against C10, when it yielded the 18 hr. plaques, with no increase on further incubation. This, of course, agrees with the fact that C7X filtrates when grown on C10, did not show the increase. An 18 hr. plaque, picked off and treated in the same manner, gave rise only to 18 hr. plaques (Table 4).

Failure to isolate a sensitive variant from strains C7X, C1 and C1R

Since phage liberation occurred at $1\frac{1}{2}$–2 hr. in cultures of C7X, i.e. at the same time as when free Px phage acted on strain C10, and because of the apparent suppression of this mechanism by the action of compounds A and B, attempts were made to isolate a sensitive variant from strain C7X. This would be expected to be difficult since, being sensitive, such a variant would normally be lysed. Culture C7X was subcultured six times in medium containing 0·25 mg. compound B/ml. to suppress lysis of the assumed variant. The culture was then diluted in Ringer's solution containing hydrogen peroxide (a $1/10^5$ dilution of an approximately 40% solution was the maximum concentration which did not affect host growth) and the dilutions held at room temperature for 3 hr. This treatment was known to eliminate most of the free phage (Dickinson, 1948). Volumes (0·25 ml.) of treated C7X culture were then spread on the surface of nutrient agar plates by a wire spreader, and similar volumes were spread on the surface of agar plates containing 1 mg. compound B/ml. to favour the selection of a variant if present. Similar counts were also made after growing strain C7X in the presence of antiserum to Px phage at a dilution of 1/10. Many thousands of colonies were examined, in particular those appearing to be non-iridescent, and about 300 colonies were subcultured into broth and tested, but all were insensitive to filtrates of C7X and were iridescent on subculture. Strain C1 and its non-iridescent variant C1R were also examined; all colonies produced phage in broth and were insensitive to filtrates of C1 and C1R.

Iridescence

Dickinson (1948) noted that iridescence was not apparent when cultures were grown on nutrient agar plates incorporating phenol, hexylresorcinol, agaric acid or proflavine; only proflavine inhibited the growth of bacteriophage. Warner (1950) reported that saponin, hexylresorcinol and other surface-active agents suppressed iridescence but had no effect on phage action. All the above-mentioned compounds, with the exception of proflavine, might be expected to disperse the iridescent material which Warner found to be a waxy substance. When these surface-active agents were incorporated in our liquid media and 24 hr. cultures plated on nutrient agar, iridescence was very marked. Proflavine at 1·0 and 0·1 mg./ml. nutrient agar inhibited iridescence of all five of our iridescent strains and, at the same concentrations, inhibited lysis of C10 by Pa, Pb and Px phages on the same plates. Proflavine was not a very satisfactory substance to use, as already explained, and compounds A and B were therefore tested. These also inhibited both the lysis of C10 and iridescence of C7X and other cultures. After eight subcultures in media containing 0·25 mg. compound A or B/ml. the cultures still produced iridescence when grown on nutrient agar, and also produced phage. Pl. 1, fig. 2, shows the colony appearance of C7X on agar containing 1 mg. compound B/ml.

Use of chemically-defined media

Ps. pyocyanea grows well in a simple chemically-defined medium consisting of lactate and salts; potent phage preparations can readily be obtained by the use of glycerol or heat (to inactivate the host cells) but not by filtration (Dickinson, 1948). Lysis on this medium solidified with agar was, however, very variable. When Px, Pb and Pa phages were spotted on C10 grown on this solid medium, with or without 1 % casein hydrolysate, there was often no apparent lysis. Organisms from the spotted area, however, were resistant to phage action when tested on nutrient agar, whereas controls from other areas on the plate were still sensitive. Don & van den Ende (1950) noted similar difficulties with certain batches of solid medium. The same batch of casein hydrolysate medium solidified with agar was used to test the action of T1 phage on *Esch. coli*; the lysis here was clear-cut and discrete plaques were quite distinct. It is possible that resistant strains grew up very rapidly on this medium as the same medium without the agar gave high phage titres.

The iridescent strains did not exhibit their iridescence on chemically-defined medium solidified with agar, but iridescence reappeared when the cultures were grown on nutrient agar. Strain C7X produced the same amount of phage in liquid defined medium as in broth. These cultures possessed an iridescent pellicle, often with a plaque appearance, and gave iridescent colonies when plated on nutrient agar. Since normal phage action was so difficult to assess on defined medium agar, the suppression of iridescence may be related to this problem.

DISCUSSION

Many hypotheses have been suggested to account for the behaviour of iridescent cultures of *Ps. pyocyanea.* Warner (1950) considered that the iridescent material was a distinct lytic entity, but it has not been proved that this material can in fact cause lysis. The evidence for Warner's view lies mainly in the action of surface-active agents and the effects of different media. Don & van den Ende (1950) noted that iridescence was suppressed on MacConkey's and Bacto S.S. media. It is possible that this suppression is due to the presence of surface-active bile salts in the media. The delayed and progressive appearance of iridescence compared to the production of plaques at 18 hr., has been previously taken as evidence for dissociating phage activity from the appearance of iridescence. However, with cultures C7X and C1, plaques increase in size and number after 18 hr. There is, therefore, no convincing evidence that iridescence is a distinct lytic entity. All these phenomena, as well as the effect of compounds which inhibit phage multiplication and the appearance of the plaque-like iridescent areas in iridescent cultures (see Pl. 1), could equally well be explained by the hypothesis that the iridescent substance is produced during the breakdown of cells lysed by phage action. As non-iridescent lysogenic strains, e.g. C1R, occur and since phage lysis can occur without iridescence, e.g. phage Px acting on strain C10, then, on this hypothesis, the iridescent material must only be produced during the destruction of certain strains of *Ps. pyocyanea.*

The mechanism of phage action in strain C7X is of interest in relation to the problem of lysogenic bacteria. Lwoff & Gutman (1950) found that apparently every cell in their strain of *Bacillus megatherium* 'carried' phage and that lysis, which produced free phage in their cultures, was precipitated by some environmental factor. A further paper by Lwoff, Siminovitch & Kjeldgaard (1950), appearing since the present work was submitted, supports this view. Boyd (1950) mentioned that in certain cirumstances, symbiotic phages of *Salmonella typhimurium* split off variants which had lost their adaptive properties and were lytic in nature.

This case of *Ps. pyocyanea* appears to be more complex than these. Don & van den Ende (1950) found that their L strain 'carried a phage or phages which were shown in several tests to be active against its own variants'. Filtrates from the metallic (i.e. iridescent) variant derived from the L strain also lysed variants. A non-lysogenic variant gave rise to lysogenic variants, and the action of phage on a non-metallic variant produced a metallic variant (this may be similar to the origin of C7X from C7 and F1).

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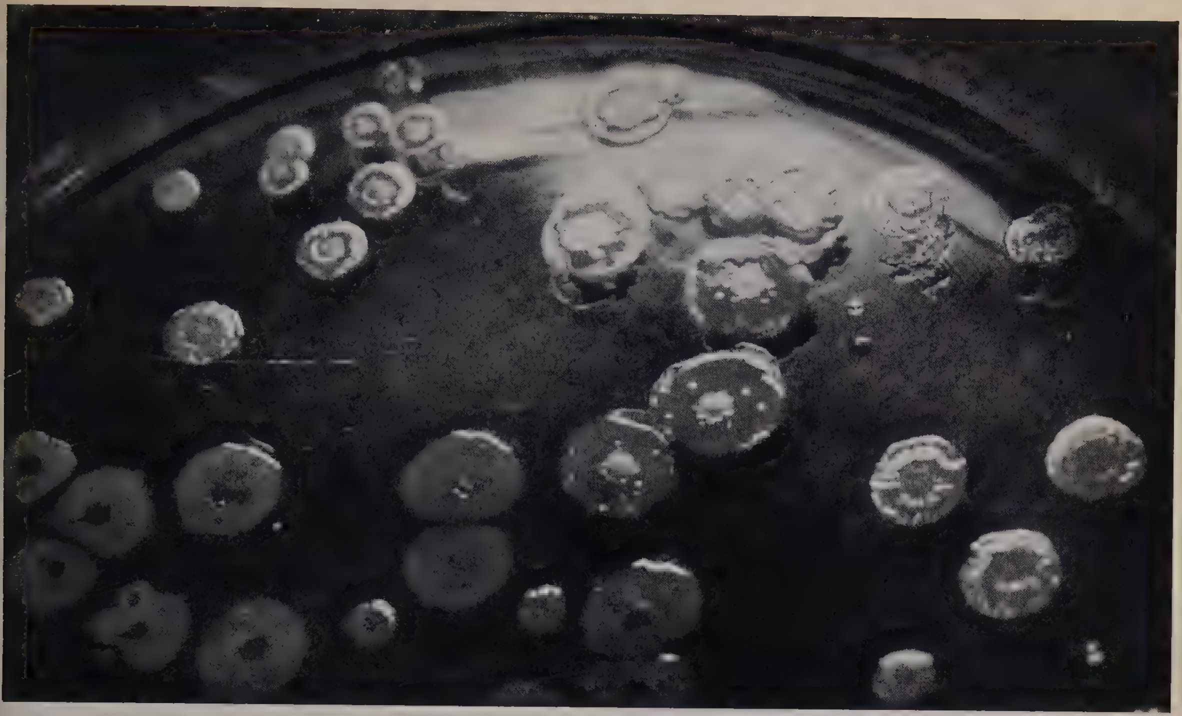

Fig. 1

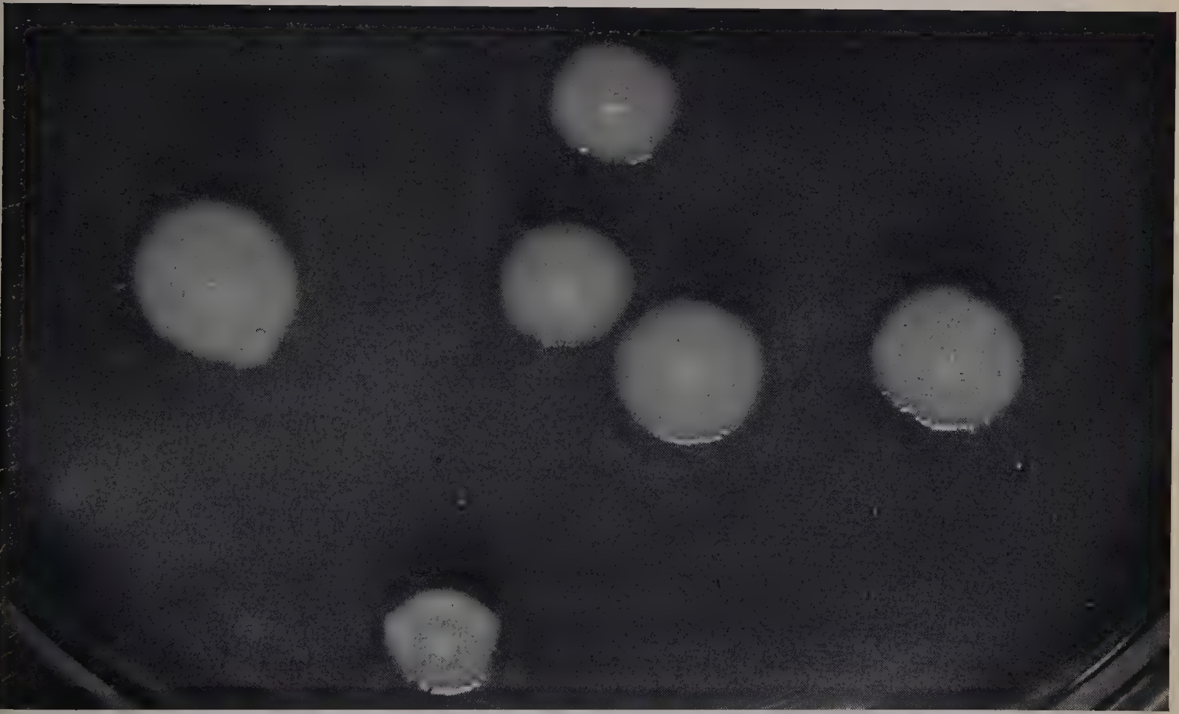

Fig. 2

Loïs Dickinson & Sheila Codd—Phage reproduction in *Ps. pyocyanea*. Plate 1

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EXPLANATION OF PLATE

Fig. 1. 48 hr. colonies of C7X. Magnification, ×2.

Fig. 2. Colony appearance of C7X on agar containing compound B. Magnification, ×2.

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A Rapid Method for the Turbidimetric Assay of Antibiotics

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SUMMARY: By using a culture of *Streptococcus agalactiae* in its logarithmic phase of growth as test organism, it is possible to assay nisin satisfactorily with an incubation period of approximately half an hour. Penicillin, streptomycin, aureomycin and gramicidin cause a qualitatively similar response in the test organism. A similar technique could therefore be used to assay these antibiotics.

A considerable number of turbidimetric assay methods have been described (see, for example, Florey, Chain, Heatley, Jennings, Sanders, Abraham & Florey, 1949). One of the most rapid of these was due to Lee, Foley, Epstein & Wallace (1944), who used a streptococcus of group B and incubated until a sufficient turbidity had developed, 'usually for 90 min. or less'. For the sake of rapidity Friedmann & Beach (1951) used *Streptococcus cremoris* in nisin assays; resazurin was employed as an indicator of bacterial growth, enabling assays to be read after 20 min. A standard error 'of the order of 10 %' was claimed for five estimations. In the work to be described *Strep. agalactiae* was preferred, since previous experience had shown *Strep. cremoris* to be sensitive to antibiotics which had little effect against pathogens.

The properties of nisin which affect its assay have been discussed by Hirsch (1950), who has also described two other methods of assay, one based on the bactericidal properties of nisin, the other on its property of prolonging the lag phase of growth of the test organism (Hirsch, 1950). The former method is also of low reproducibility, but the latter has an accuracy varying from ±7 to ±15 %, and is therefore far more accurate than any of the earlier methods. However, a rapid assay was desired and a comparison of Fig. 2 in the paper by Pope (1948) with the work of Bond & Davies (1948) suggested the method described below.

The present assay is simply carried out by making dilutions of unknown samples in powers of 2 in 0·01 N-HCl, including in each batch at least one set of tubes containing a standard solution diluted in powers of 1·5, 'inoculating' each tube with six volumes of a culture in the logarithmic phase of growth, and after 30 min. incubation, halting the growth and determining the optical densities of the appropriate suspensions. Graphical comparison in the usual way between unknown and standard tubes gives the nisin content of the former.

METHODS

Definitions. (*a*) Units; see Mattick & Hirsch (1947) and Berridge (1949). One unit normally delays the growth of *Strep. agalactiae* in 1 ml. of broth for about 16 hr. (*b*) Optical density (O.D.); this is the difference in Spekker readings on the logarithmic scale between clear broth and the culture being examined. A 4 cm. cell was used.

The cleaning of glassware. This was done by soaking overnight in 0·01 N-NaOH which destroys traces of nisin adsorbed by the glass.

Maintenance of culture. Stock cultures of *Strep. agalactiae* were kept in bullock heart medium. Cultures for daily use were propagated in Y.G.P. (Yeastrel glucose peptone broth) twice daily. This, it is believed, helped to maintain a rapidly growing strain. During week-ends the culture was refrigerated. Cultures freshly taken from stock were sometimes unsatisfactory as to growth rate, but daily passage for about a week through bullock heart medium restored each culture to normal.

The medium for assays. Yeastrel glucose broth, glucose Lemco broth and the medium of Bond & Davies (1948) were all found satisfactory. Medium to be used on the day of working was merely steamed and filtered, the usual autoclaving being omitted. The complete coagulation of protein and efficient filtration are, however, important.

The preparation and maintenance of a culture in the logarithmic phase of growth. Each evening 10 ml. of Y.G.P. was inoculated with a loopful of 7–8 hr. culture, and placed in cold tap-water (10–15°) in an apparatus which raised the temperature to 40° after about 10 hr. A time switch and thermostat were used on some occasions; on others 300 ml. of the cold water were placed in a litre vacuum flask in an incubator. In the morning the culture (10 ml.) was added to 100 ml. of warm Y.G.P.; this was further diluted while the streptococci were still actively growing, and within 2 hr. a litre or two of broth was ready for assays. Aseptic precautions were not observed beyond this stage. The culture was kept in its logarithmic phase merely by diluting before the optical density reached its maximum. This was at first controlled by Spekker readings, but since there is a wide margin of safety a little practice permitted it to be done by eye. Thus, when the culture looked dense enough (O.D. 0·5) it was diluted 10- to 20-fold and could be left for an hour.

The culture for assays. The logarithmic phase culture was diluted by stages, keeping the O.D. between 0·02 and 0·05 until the volume required was obtained, and was finally adjusted to an O.D. of between 0·17 and 0·25 just before use.

Diluting the antibiotic. This process was carried out in a modified 5 ml. syringe. A stop, e.g. a 'Meccano' collar or a pile of washers, was fitted on the stem just above the plunger so that the capacity of the syringe was limited to 5·0 ml. A spring was fitted between the barrel cover and the handle of the stem so that its force raised the plunger with a brisk motion to its highest point. This spring was cut to such a length that when it was completely compressed the plunger reached only the 2·5 ml. mark. A suitable spring was made from a standard 'Meccano' spring by pulling it out and cutting off the required length. The syringe was first filled with 0·01 N-HCl, the plunger brought to the 2·5 ml. mark and 2·5 ml. of the antibiotic solution were sucked in; 2·5 ml. of the mixture were then ejected into the first assay tube and the operation repeated as required. Beginning with a nisin predilution containing 4·0–200 units/ml., seven such doubling dilutions were made. The cycle of dilution followed by emptying, rinsing and refilling the syringe can be completed in 47 sec. An allowance of 1·5 min. per assay for this part of the work thus allows time for careful operation.

With the syringe technique there is a risk of contaminating with nisin the bulk of the broth used for dilution every time the syringe is put into it. Contrary to expectation this contamination proved negligible in practice. Experiments with coloured solutions showed that with a smooth technique of normal speed the contaminated solution near the tip of the syringe was sucked into the syringe on the upward stroke. The precautions were therefore limited to the use of a small beaker for the diluting medium, emptying and rinsing the beaker once, and the syringe twice between each set of dilutions. The traces of nisin which remained on the sides of the plunger seemed to be insufficient to affect the assay. Titrations confirmed that an acid could be diluted accurately by this technique.

Adding the test culture. Into each tube 15 ml. of the culture at 40° were rapidly pumped with sufficient tangential force to wash the sides and to ensure thorough mixing without foaming. A hand-operated ampoule-filling machine (A. J. Manning Ltd., Wembley) enabled 100 tubes to be filled in less than 3 min.

Incubation. Bond & Davies (1948) found *Staphylococcus aureus* in penicillin to be sensitive to minor fluctuations of temperature. The response of *Strep. agalactiae* to nisin does not show such a sensitivity so that special precautions were not required; any temperature between 37 and 44° may be used, but 40° was preferred as it gave the maximum growth rate. Half an hour was a convenient short period for incubation. On the few occasions when 1 hr. has been used, a greater slope has been obtained, but this does not necessarily give an increase in accuracy (Wood, 1948).

Halting the growth. The addition of 5 ml. of 0·004 % thiomersalate (ethyl mercurithiosalicylate) halted the growth immediately, giving a mixture which maintained a constant turbidity for about 24 hr. The thiomersalate must be injected with sufficient force to cause immediate and thorough mixing. This was done with the ampoule-filling machine, but using a finer jet than that used for filling.

Measuring the optical density. A Spekker absorptiometer was used with an Ilford light filter no. 608 and the 4 cm. cell. Because of the long chains of streptococci, turbulence in the cell caused irregular readings. An inconveniently long time was needed for turbulence to disappear, and therefore two baffles, consisting of ill-fitting glass plates at right angles to the light beam, were cemented into the cell leaving small gaps here and there around the edges. In this way turbulence was eliminated and measurements could be made without delay.

Interpretation

The optical densities in the tubes containing the standards were plotted against the nisin concentrations, and the best straight line drawn by judgement, as exemplified in Figs. 1–4, to give a graph from which the unknown nisin concentrations could be read. The straight line usually extended over a three-fold range of nisin concentration, beginning most frequently at *c.* 2·5 units/tube, though occasionally starting as low as 1 or as high as 4 units/tube. For the special purposes of Figs. 2–4 dilutions in powers of 4/3 were used. Normally powers of 3/2 were used in the standard series.

With a series of dilutions for each unknown it was often possible to obtain two readings for each nisin solution, corresponding to two levels of the standard. With solutions similar to the standard these two readings agreed. When, as frequently happened, there was disagreement arising from the presence of different antibiotics for which no standard was available, it was a matter of definition as to which level should be taken to indicate equality. The level corresponding to the range 3·5–7·0 units/tube was chosen. In order to achieve some replication in one batch of assays, it was possible to tabulate the values for the unknowns falling in the preferred range alongside the value for the tubes in the next higher or lower range (according to which extra point fell in the usable range of the standard graph), and thus to calculate for each unknown the factor relating the concentration in the high or low range to that in the preferred range. By doing this with a whole batch of, say, twenty assays of a particular preparation of nisin and taking the average for each of the two kinds of factor, it was possible to reach a presumably reliable average for each factor. Then, using this average in place of the expected 2·0 or 0·5, the values in the preferred range could be calculated from those of the lower or higher ranges. Thus duplicate and sometimes triplicate values could be obtained from one assay. Ratios varying from 1·78 in one case to 2·18 in another were obtained.

RESULTS

With nisin

A series of twenty-five consecutive duplicated assays done on several different days (during work on counter current distribution) was subjected to the analysis of variance as described by Patterson (1939). In order to avoid mixing very large with very small values, the figures as derived for the total number of units present in the chosen assay tubes were used. It seems to be the general experience that agreement of such assays on any one day is relatively close, but that variations from day to day can be great. Therefore instead of replicating tubes during each assay, the whole assay was repeated on a different day. The standard deviation therefore includes day-to-day variations. The values ranged from 2·9 to 7·2 units/tube, the mean being 4·64. The standard deviation was 0·42 unit or 9·0 % of the mean.

In a similar range of assays of crudest nisin, in which the duplicates were done on the same day, the standard deviation was 5·9 % of the mean.

In a third set of thirteen samples, nine were duplicated immediately after the first set had been diluted, and all twenty-two assays were made with the same culture. The nine duplicates gave consistently lower figures than the corresponding values in the first set, the average difference being 0·3 unit/tube, i.e. about 6 %. By allowing for the difference in 'slope' (as described under '*Interpretation*') so that two readings were obtained for each assay, and by adding the constant difference of 0·3 unit to each duplicate, it was possible to decrease the standard deviation in this series to 3·6 % of the mean. Under these conditions, therefore, duplicate assays are sufficient to discover differences greater than 10 % ($t=2{\cdot}26$ at $P=0{\cdot}05$ for 9 degrees of freedom).

Table 1. *Standard deviations of rapid assay under various conditions*

Antibiotic	No. of different samples	No. of replicates from each sample	Range of variates	Standard deviation as % of general mean	Notes
Nisin	25	2	2·9–7·2	9·0	Duplicates assayed on different days
Nisin	12	2	1·8–7·7	5·9	—
Nisin	9	2	4·1–7·8	3·6	Allowance made for differences in 'slope' and for time between duplicates
Nisin	1	10	—	4·8	Incubation: 40 min.
Penicillin	1	9	—	4·7	Incubation: 30 min.
Penicillin	1	10	—	2·2	Incubation: 60 min.

It has occasionally been said that solutions of nisin deteriorate. In our experience such deterioration has been the result of mould growth of a character easily to be mistaken for a slight precipitate. The following figures, which are not exceptional, indicate the stability of standard solutions. An approximately 1 % solution of standard powder in 0·05 N-HCl had, when fresh, an activity of 16,500 units/ml. by definition. It was kept in the refrigerator (0–2°) for 8 months, and when compared with a fresh solution of standard its activity was found to be 16,200 units/ml.

Application to other antibiotics

Figs. 1–4 show the relation between dilution and optical density when different antibiotics were tested by the technique here described. For each antibiotic a different scale was used such that the graphs approached or coincided at about 30 % inhibition. The different ages of the antibiotic samples made comparison on a weight basis useless. The similarity of form between the curves indicated that the method would probably be applicable to all these substances. In spite of the lower slope of the graph for penicillin it could be assayed using 30 min. incubation with about the same reproducibility as nisin. When, however, penicillin assays were incubated for 1 hr. starting at half the usual optical density, the graph became much steeper and the standard deviation was considerably decreased.

The results are collected in Table 1.

DISCUSSION

The effect of time noted in the last experiment with nisin described under 'Results' is small and irregular. It frequently appeared less strongly between duplicate runs with standards, and needs further investigation. This is one example of several ill-defined effects which combine to make the assay of nisin difficult. They are usually ascribed to its surface activity, since it is readily adsorbed and tenaciously held by a wide variety of substances (Berridge, 1949). This probably explains an experience with inadequately steamed broth which contained a slight haze and gave erratic results.

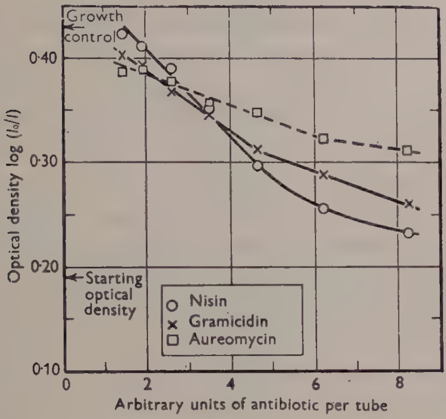

Fig. 1

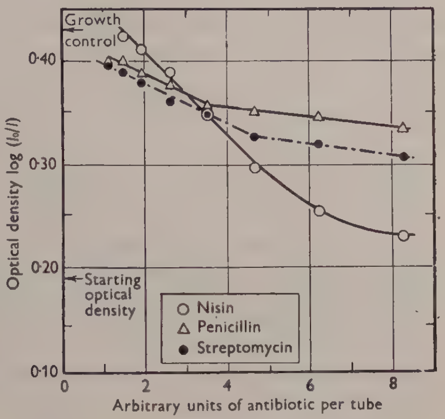

Fig. 2

Figs. 1 and 2. Response of *Strep. agalactiae* to different antibiotics; incubation period 30 min.

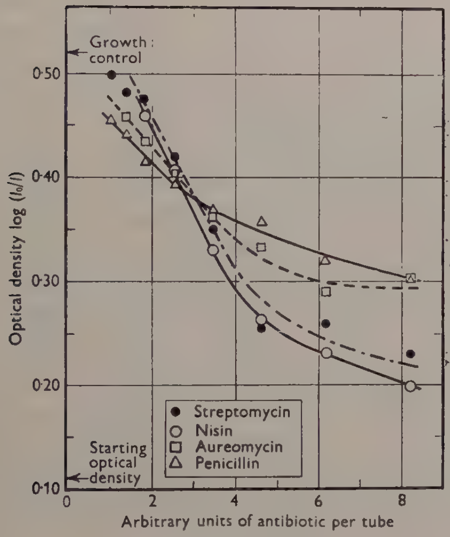

Fig. 3

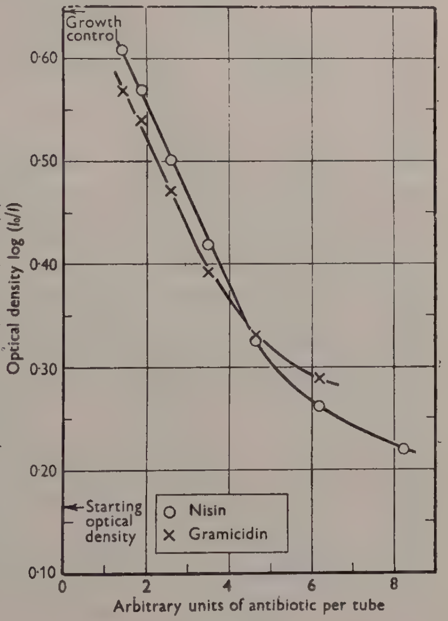

Fig. 4

Figs. 3 and 4. Response of *Strep. agalactiae* to different antibiotics; incubation period 60 min.

This technique, with minor modifications, has been used with satisfaction for about 2 years. A short incubation period was preferred in the first place because results were desired with the minimum of delay. There is, however, an *a priori* reason for expecting short incubation periods to give more accurate results, for the moment a culture of micro-organisms is divided, fortuitous causes begin to operate and to cause divergencies. The shorter the time between the division of the culture into separate assay tubes and the final readings, the less these divergencies should be. The less also will be the turbidity differences upon which the assay depends. There will thus be an optimum incubation time which may depend on the test organism, the antibiotic and the conditions of assay. It was not the purpose of this work to discover the optimum, but to show that approximately half an hour is, in general, sufficient and allows satisfactory assays to be made quickly.

Our best thanks are due to Miss S. Lamb for technical assistance.

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Inhibition of Bacterial Growth by Bacteriophage as distinct from Lytic Action

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SUMMARY: Certain races of streptococcal phage with a normal lytic action on some strains of streptococci were found to be adsorbed on, and to inhibit the growth of, other strains, without the occurrence of lysis or multiplication of the phage. One race of phage inhibited the growth of several streptococcal strains which had hitherto been considered unrelated to one another on the basis of their reactions to lytic phages. Cultures which recovered from temporary inhibition by phage contained variants which were resistant both to inhibition and to lysis by phages which attacked the original strain. This resistance is, however, short-lived as compared with the resistance of variants obtained through the action of a lytic phage.

The typical interaction between bacteriophage and bacterium is that in which adsorption of phage is followed after an interval by lysis of the bacterium and a many-fold increase in number of phage particles. The majority of workers now visualize phage as a bacterial parasite which grows in or on the bacterial cell, ultimately causing lysis with liberation of phage particles. There are, however, other types of association between phage and bacterium to which this picture does not apply. For instance there are the cases where bacteria are lysogenic, or where resistant organisms support the growth of an associated phage. Another association which is not followed by lysis or phage reproduction has also been observed. Rakieten & Rakieten (1937) and Rakieten & Tiffany (1938) observed that staphylococcus phages were absorbed on strains of *Bacillus subtilis* and of enterococci without apparent effect on the growth of these organisms. Andrewes & Elford (1932) and Adams (1949) reported observations on 'citrate sensitive' coli phages which, in the absence of calcium, attached themselves to bacteria, prevented bacterial multiplication, but did not cause lysis. Felix (1949) reported that with certain salmonella adsorption of phage was not always followed by lysis.

We have encountered in the group of lactic streptococci some instances of phage adsorption without subsequent lysis. Certain phage races which exert a lytic effect on some strains of streptococci are adsorbed by other strains without any lytic effect and when present in relatively high concentration the adsorbed phage inhibits the growth of the adsorbing organisms. It appears that adsorption takes place as usual but that subsequent growth of the phage, normally leading to lysis, is blocked. This blockage also results apparently in interference with the metabolism of the bacterium.

MATERIALS AND METHODS

Cultures. The eight bacterial cultures used were strains of *Streptococcus cremoris* as used in commercial cheese manufacture. They had been isolated in this Institute over about 17 years, and were selected on the bases of acid-producing activity in milk and lack of relationship to one another in lytic phage reactions. The stock streptococcal cultures were maintained at 22° in sterilized milk and transferred daily. Although these strains grew more rapidly between 30° and 35° during one or two subcultures, they rapidly lost their acid-producing power when maintained any longer at such temperatures and died out after transfer for a few days at 37°.

Demonstration of phage action. The stock phages, originally isolated from cheese whey, were maintained as whey filtrates (Seitz) at 5–7°. The demonstration of phage action on a bacterial culture (*a*) and the estimation of the strength of phage preparations (*b*) respectively, were carried out as follows. (*a*) Standard loopfuls (0·004 ml.) of 1/10 dilutions were added to tubes (9 ml.) of sterilized skim milk inoculated with two drops (0·1 ml.) of a 24 hr. culture of the required streptococcal strain and incubated at 30° or 37°. All tubes which showed coagulation in 24 hr. were subcultured once. (*b*) By means of a standard loop a drop of each 1/10 dilution was placed on the surface of an agar plate previously spread with a mat of the streptococcal culture, and incubated at 30°.

The phage titre is expressed as the highest dilution which caused failure to coagulate in method (*a*) or which gave plaques in method (*b*). Frequently method (*a*) gave a titre higher by one dilution than method (*b*). This was to be expected since streptococcal phages form small plaques which when present in small numbers, can easily be overlooked. In spite of this we generally used method (*b*), which was accurate enough for our purposes, had the advantage of revealing anything characteristic about plaque form or size, and lent itself to diagrammatic presentation of results.

Phage adsorption. Five ml. of a 24 hr. clotted milk culture of the streptococcal strain under test were measured into a sterile 4 × ½ in. tube fitted with a rubber bung. Two drops (0·1 ml.) of undiluted phage preparation were added and the mixture allowed to stand at room temperature for 5 hr. with frequent shaking. It was then centrifuged, the clear supernatant fluid drawn off, a series of 1/10 dilutions prepared from it, and the phage titre determined by the plate method (*b*). A control series of dilutions starting from two drops of the phage preparation in 5 ml. saline was also spotted on the plate. (This control gave the same titre as a control prepared from two drops of phage in 5 ml. of acid-clotted skim milk). Where a series of cultures was tested for ability to adsorb a certain phage, and the supernatant fluids were spotted on a plate spread with the culture which the phage normally lysed, adsorption of phage was made evident by a significant decrease (at least by two dilutions) in titre of the supernatant fluid from the adsorbing cultures. In this test the homologous culture always showed adsorption of phage. Cultures not lysed by the phage showed adsorption of various degrees or gave a result identical with that of the control, indicating no adsorption.

Observation of lysis or inhibition under the microscope. Tubes of sterilized milk inoculated with streptococcal strains and with various amounts of phage were incubated in a water-bath at 30° or 37°, the temperature selected depending on the characteristics of phage race and bacterial strain. At intervals, smears from the tubes were made on a slide, dried, and stained with methylene blue. By examination under the microscope an estimate was made of extent of growth, progress of lysis, and any other effect which the phage might have on the morphology or arrangement of the bacteria. It was adequate for our purpose to use an estimate based on inspection of a series of fields and expressed by signs ranging from – to + + + +.

RESULTS

A strain of *Streptococcus cremoris*, designated D4, was received from Australia during a search for active acid-producing cultures for use in cheese manufacture. After the strain had been in use for 6 weeks in a commercial cheese factory, a phage capable of lysing the organism was regularly found to be present in the cheese whey. This was in accordance with normal expectation and, we believe, implies that the phage, present in dormant form in infinitesimal amount in the factory surroundings, gradually increased in concentration by multiplication on the susceptible organism which was grown daily as a large exposed culture in the cheese vats. Phage 51 isolated in this way had a normal lytic action on *Strep. cremoris* strain D4. Tested on agar against seven other 'unrelated' strains of *Strep. cremoris*, a preparation of phage 51 which had a titre of 10^{-8} against streptococcus D4 did not show clear areas of lysis or plaques on any of the cultures; but on some of them it produced foggy areas of decreased growth where the drops of undiluted phage had been placed. Trial of the action of this phage on the cultures in a skim milk medium showed that there was inhibition of growth of those cultures which had shown the foggy areas in the plate test.

Table 1 gives the results of an experiment in which the seven *cremoris* strains were treated with varying amounts of phage 51, the extent of growth being estimated under the microscope as described. The results indicate that four of the strains were markedly inhibited by phage 51, two were only slightly affected and one was not influenced by the largest amount of phage used. The inhibitory action was usually only temporary; with smaller amounts of phage, cultures tended to recover within 6 hr. from the start of the experiment, and milk was usually coagulated overnight as with a normal culture. With larger amounts of phage, growth sufficient to cause coagulation of milk occurred only after two or three days. This inhibitory action of a single phage on several of the strains of *Strep. cremoris* in this group was all the more unexpected since these strains had been selected because of their apparent complete lack of relationship to one another in respect of sensitivity to lytic phages. In no instance, over the course of years, had a phage with lytic action against one of these strains been found capable of lysing any of the others. It should be noted, however, that the amounts of phage used in the above test were much higher than would normally be used in testing a phage for lytic power; subsequent trials, mentioned later, showed that among the lytic phages active on these seven strains were some which had inhibitory activity against heterologous strains.

Table 1. *The effect of phage* 51 *on the growth after* 5 *hr. at* 30° *of seven strains of* Streptococcus cremoris

	Vol. of phage 51 preparation added to 9 ml. milk culture (ml.)				
	Nil	0·1	0·25	0·5	1·0
Strain of *Strep. cremoris*	Relative amount of growth at 5 hr.				
HP	++++	+++	−	−	−
K	++++	+++	+	−	−
R1	+++++	+++	+	−	−
R6	++++	−	−	−	−
ML1	++++	++++	++++	++++	++++
KH	+++++	+++++	+++++	+++++	++
E8	++++	+++	+++	++	+

Nature of the inhibitory principle

The question arose whether the inhibitory action of the phage preparation was due to the phage itself or to some antibiotic substance formed during the growth or lysis of streptococcus D4. The question was answered in favour of the first alternative by the following observations. (i) The inhibitory principle was destroyed by heat coincidentally with the phage. Exposure to 66° for 5 min. (pH 5·0) was sufficient to destroy both lytic and inhibitory power in the whey filtrate. (ii) Phage 51 prepared by lysis of a strain of *Strep. lactis* (the only other organism apart from the streptococcus D4 so far found to serve as host) had exactly the same inhibitory power as phage 51 prepared by lysis of *Strep. cremoris* strain D4. (iii) Another phage race active against streptococcus D4 in a normal lytic manner did not show any inhibitory action against the other strains of *Strep. cremoris*. It seems most probable therefore that the inhibitory action was due to phage 51 itself and did not depend on a property of streptococcus D4.

Appearance under the microscope

Tubes containing 9 ml. of sterilized skim milk were inoculated with 0·1 ml. of a 24 hr. milk culture of streptococcus HP, and amounts of a preparation of phage 51, ranging from 0·05 to 1 ml. in the various tubes, were added. The tubes were incubated at 30° and smears made at intervals for staining and microscopical examination. A minimal amount of phage sufficient to have an observable inhibitory effect within the first 5 hr. of incubation (*c.* 0·05 ml. of a preparation with a titre of 10^{-8} on streptococcus D4) caused a decrease of growth as compared with a control and a shortening of the chains of streptococci. With increasing proportions of phage there was progressively less growth in the cultures and, as well as the shorter chains, there was an appearance as though individual cocci detached from the chains and lost their normal spherical shape. Tubes to which 0·5 ml. of phage preparation had been added frequently showed after 5 hr. incubation little more than a few of these 'spots', which gave the impression of being dead cocci in process of dissolution. The picture under the microscope thus suggested a drastic interference with the metabolism of the bacteria, the degree of interference depending on the number of phage particles present. On continued incubation the cultures containing the smaller proportions of phage showed complete recovery within 24 hr. Those containing larger proportions showed little growth after 24 hr., but they grew sufficiently to cause coagulation of the milk after a few days.

Adsorption of phage on bacteria

When a culture of streptococcus HP, after being initially inhibited by phage 51, recovered and finally clotted the milk, it was possible to show that some adsorption of phage had taken place. The supernatant fluid obtained by centrifuging such cultures, containing originally up to 0·5 ml. of phage, did not contain enough phage to give plaques on a plate spread with streptococcus D 4. Cultures to which more than 0·5 ml. of phage had originally been added showed some phage in the supernatant fluid. A demonstration of phage adsorption by various strains of *Strep. cremoris* was more conveniently carried out, however, by the method described earlier. Clotted milk cultures of the seven streptococcal strains used were investigated for their capacity to adsorb phage 51. The results are shown diagrammatically in Fig. 1. The results indicate that the strains most powerfully inhibited adsorbed the phage most readily. There were some anomalies with the three strains ML 1, KH, and E 8. Strain ML 1 was not significantly inhibited by 1 ml. phage preparation per 9 ml. of culture, yet it adsorbed phage as strongly as strain E 8 which suffered moderate inhibition. Strain KH adsorbed more strongly than strain E 8 yet was inhibited to a lesser extent. In general, however, it is reasonable to conclude that adsorption of phage and inhibition of growth were linked.

When the test was applied to organisms and phages whose combination resulted in lysis, adsorption was always evident, as with streptococcus D 4 in Fig. 1. The adsorption was always less marked, however, than it was with organisms which were strongly inhibited. It cannot be deduced from the adsorption tests with clotted milk cultures whether an adsorbing organism will show lysis or inhibition by the phage.

The results obtained in an adsorption with a phage race which had no inhibitory action on the *cremoris* strains used are illustrated in Fig. 2. which should be contrasted with Fig. 1. Phage race 2 had a lytic action on streptococcus HP only, and the results show that adsorption to a significant degree occurred only with streptococcus HP.

Adsorption tests carried out with the *cremoris* strains and the various phage races which attacked them disclosed two more instances where phages had an inhibitory action on strains other than those which they lysed. Phage 4 which lysed streptococcus K was adsorbed on and inhibited streptococcus R 6. Conversely phage 9 which lysed streptococcus R 6 was adsorbed on and inhibited streptococcus K. These reactions had been overlooked when tests for phage action had been carried out with only small amounts of phage and adsorption tests had not been done. It seems evident that the phenomenon of adsorption and inhibition of growth without lysis is not uncommon among phages for the lactic streptococci, although the action of phage 51 on unrelated strains may be unusual.

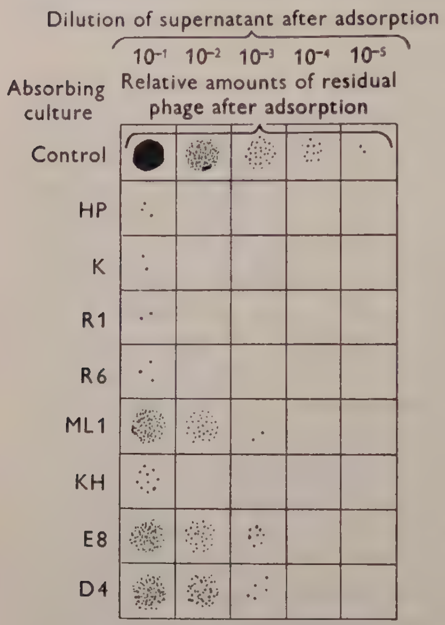
Dilution of supernatant after adsorption
10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵
Absorbing culture
Relative amounts of residual phage after adsorption
Control
HP
K
R1
R6
ML1
KH
E8
D4

Fig. 1

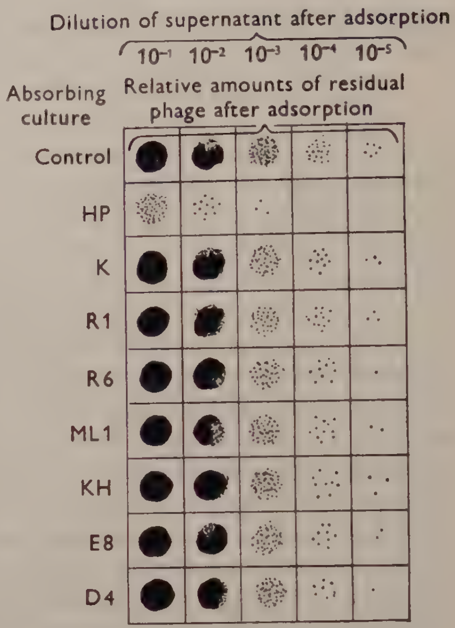
Dilution of supernatant after adsorption
10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵
Absorbing culture
Relative amounts of residual phage after adsorption
Control
HP
K
R1
R6
ML1
KH
E8
D4

Fig. 2

Production of phage-resistant variants

The bacterial growth which eventually occurred in a milk culture in which streptococcus HP had temporarily been inhibited by phage 51 differed from a normal culture of HP in that it frequently contained phage-resistant variants. Tubes of skim milk inoculated with HP, and various amounts of phage 51 were incubated at 30° until the milk clotted; the resulting cultures were streaked on agar. Colonies picked at random from the incubated plates into sterile milk yielded a series of subcultures, some of which were apparently identical with the original strain HP while others differed in appearance under the microscope and in reaction to phage. These variants were much less inhibited by phage 51 than was a normal culture of HP and were completely resistant to phage 2, a race which lysed HP. The proportion of phase-resistant variants in the cultures was not constant from experiment to experiment; the maximum proportion was estimated at 20 %.

Variants obtained as a result of the action of phage 51 were compared with phage-resistant variants obtained in the usual way by continued incubation of a culture of streptococcus HP lysed by phage 2. The latter treatment yielded a large proportion (often 100 %) of resistant substrains which are considered (Luria & Delbrück, 1943; Delbrück, 1946) to have originated from a few resistant individuals present in the culture as a result of a normal process of variation. The two kinds of resistant streptococcus, namely that produced during inhibitory action by phage 51 and that developed after total lysis of a culture by phage 2, appeared to be similar. They differed from the normal streptococcus HP in appearance under the microscope, the cocci being smaller in size and the chains being much longer. The appearance was indeed so characteristic in cultures incubated at 30° that it was possible to predict, from appearance alone, which of the substrains derived from cultures grown under the influence of phage 51 were resistant to lysis by phage 2 and which were not. The results of growth tests of representative substrains of the two kinds of streptococci subjected to the action of phages 2 and 51 are given in Table 2.

Table 2. *Effect of lytic and inhibitory phage on resistant variants of streptococci at* 30°

		Streptococcus strain					
		HP		HP/2		HP/51	
Addition		Periods of growth					
Phage	Vol. (ml.)	4 hr.	6 hr.	4 hr.	6 hr.	4 hr.	6 hr.
		Relative amounts of growth					
	0	+++	++++	+++	++++	+++	++++
No. 2	0·005	−	−	+++	++++	+++	++++
No. 51	0·10	+	+	+++	++++	+++	++++
No. 51	0·25	−	−	++	+++	++	++++
No. 51	0·50	−	−	++	++	++	+++
No. 51	1·00	−	−	+	+	+	+

HP/2 Resistant substrain prepared by action of lytic phage 2.
HP/51 Resistant substrain prepared by action of inhibitory phage 51.

It is clear from the results that both types of variants were completely resistant to lysis by phage 2 and that both, although slightly affected by phage 51, were much less inhibited by that phage than was the original strain HP. At first it seemed that the two resistant strains were identical, and that a close linkage existed between the action of the two phages, one lytic and the other inhibitory. But it soon became evident that there was a difference between the streptococcal strains HP/2 and HP/51. Whereas most strains of HP/2 retained their resistance to phage 2 at least for several months and sometimes for years, only gradually reverting to sensitivity, all the strains of HP/51 so far investigated reverted to a state sensitive to both phages within 14 days of daily subculture in milk. This fact, coupled with the early appearance and large proportion of HP/51 forms found in a culture temporarily inhibited by phage 51, strongly suggests that HP/51 variants are the result of a temporary change impressed on the bacteria by the action of phage 51, whereas HP/2 forms are probably variants normally present in the culture and made evident by the removal, by lysis, of all sensitive forms.

DISCUSSION

The inhibitory action of phage 51 on several strains of streptococci seems to be bound up with the formation of a host-virus complex in which further action leading to lysis is blocked. We have no evidence to indicate whether the blockage results from the presence of an inhibitory factor or the lack of some essential factor in the medium (cf. the 'citrate sensitive' *coli* phages), or whether lysis is impossible under any circumstances. The fact that relatively large amounts of phage (as compared with the amounts commonly used with a lytic phage) must be added to a culture to produce marked inhibition accords with expectation, since it is reasonable to suppose that the attachment of at least one phage particle is necessary to inhibit growth of each bacterium. With a lytic phage, lysis of the whole culture could, under ideal conditions, result from the presence of one phage particle by a series of waves of lysis following multiplication of phage and infection of further bacteria. In the inhibition phenomenon, where no multiplication of the phage takes place, the quantitative relationships are different.

The observation that phage 51 is adsorbed on and inhibits the growth of several strains of *Strep. cremoris* which are susceptible to attack only by distinct lytic phages raises an interesting point. The apparent lack of relationship between the lytic strains seems to indicate that the points of attachment on the bacteria for the lytic phages are distinct and unrelated; a lytic phage for one strain finds no point of attachment on the others and hence is not adsorbed. Yet several of the strains evidently have some point of attachment for adsorption of phage 51, which finds points of attachment on streptococcus D4 and on a strain of *Strep. lactis* which lead to lysis. It is possible, therefore, that the organisms which suffer inhibition of growth have more than one point of attachment for phages because they have points for the specific lytic phages and a common point for the inhibitory phage 51.

Another question concerns variants of streptococcus HP which become able to grow in the presence of phage 51 after having overcome its inhibitory effect and which are also resistant to the lytic phage, race 2, which normally attacks streptococcus HP. Also, variants of streptococcus HP resistant to phage 2, isolated from a lysed culture of HP, are resistant to the inhibitory action of phage 51. This suggests that the point of attachment on streptococcus HP for the lytic phage 2 is also involved in the attachment of inhibitory phage 51. This finding needs to be reconciled with the findings detailed in the previous paragraph. The fact that resistant forms of streptococcus HP, after the action of phage 51, rapidly revert to sensitivity to both lytic and inhibitory phages still further complicates the picture. Resistant streptococci obtained after the action of a lytic phage are almost certainly variants normally present in the culture in small numbers and made apparent because of the complete elimination of all the phage-sensitive forms. Resistant streptococci obtained by the action of inhibitory phage 51 occur in relatively large numbers in a culture which has only been partially inhibited. This suggests that these streptococci are the result of a change impressed on individuals in the culture by the action of phage. The change appears to be transient since the drift back to sensitivity is usually complete in 10–14 days.

In this relationship between variants produced by the action of lytic and inhibitory phage there is a suggestion that adsorption of an inhibitory phage, which obviously results in interference with some metabolic activity of the adsorbing bacterium, is related to the primary action of a lytic phage. This, if substantiated, would be evidence that the lytic phage acts by interposing itself at some point in the essential metabolic machinery of the organism before beginning to multiply.

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The Assay of Micrococcin, an almost Insoluble Antibiotic

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SUMMARY: The difficulties of assaying micrococcin by the usual microbiological methods and means of obviating or decreasing some of them are described. Certain detergents have a solubilizing effect on micrococcin and it is possible to assay such 'solutions' by hole-plate or other diffusion methods which cannot otherwise be applied. Low results are obtained by this method in crude fermentation liquors, probably because some of the micrococcin is present in a bound form. Ethanolic solutions of micrococcin have a powerful purple fluorescence in ultraviolet light by which amounts of the order of 2·5 μg./ml. may be accurately estimated. Details are given of the procedure, and of the special precautions necessary when micrococcin in broth or tissue is to be estimated. Micrococcin may also be estimated by measuring the absorption in ethanolic solution at 345 mμ., at which wavelength $E^{1\%}_{1\,\text{cm.}} = 177{\cdot}5$.

Micrococcin was first described by Su (1948), who investigated several microbiological methods for its assay. These are complicated by (1) the low solubility of micrococcin in water (of the order of 1 μg./ml.), (2) the ease with which bacteria become resistant to it, and (3) its relatively high molecular weight. The present paper describes further work on microbiological methods (mainly at Clevedon) and on physical methods (at Oxford); a third section describes the use of detergents in the diffusion assay of micrococcin, a technique which may be applicable to other relatively insoluble antibiotics. Pure micrococcin (Heatley & Doery, 1951) was used throughout, except in the experiments with crude micrococcin-containing culture fluid.

GENERAL MICROBIOLOGICAL METHODS OF ASSAY

Diffusion methods. The paper disk, hole-plate, cylinder-plate and similar methods are unreliable, because a workable zone diameter is only given by supersaturated solutions; the conditions governing the degree and duration of supersaturation are obscure. In addition, the curve relating zone diameter to concentration is unusually flat, each doubling of concentration resulting in an increment of 0·6–0·9 mm. in the zone diameter. Nevertheless, Su (1948) found the method useful for semi-quantitative assay of relatively low potency culture fluids.

The direct diffusion assay of micrococcin in organic solvents by dipping a paper disk into the solution and allowing it to dry before placing on a seeded plate was also reported by Su. Although it has given good results with some antibiotics, this method has since been found to be particularly unreliable for micrococcin.

Dilution methods. Since suitable test organisms are inhibited by concentrations of micrococcin well below that in a saturated watery solution ($< 1\,\mu$g./ml.), the first difficulty mentioned for diffusion assay does not arise. Su showed, however, and it has since been confirmed, that resistant mutants are readily formed in a single step or may be present initially, so that dilution tests in *fluid medium* are unreliable.

The solid medium dilution test (Su, 1948) is more satisfactory and is still perhaps the method of choice. Double strength nutrient agar was mixed with serial dilutions in water of the sample to be assayed. The plugged tubes were autoclaved, mixed, and allowed to cool in an inclined position. The surface of each slope was then flooded with a suspension of spores of the test organism and the excess inoculum drained by inverting the tube in a large test-tube where it was left during overnight incubation at 37°. In his later work Su covered the whole rack, containing up to eighty tubes, with a metal tray and inverted it as a unit. In using Su's method the test organism he specified (*Bacillus subtilis*, G8B) was not available, but satisfactory results have been obtained with either of the following inocula:

(1) One or two drops of a 4–6 hr. broth culture of '*B. subtilis*' NCTC 6346. This culture, which was almost certainly a strain of *B. cereus* (K. Crawford, personal communication), was usually inhibited by 0·03–0·1 μg. micrococcin/ml.

(2) One-fifth of a millilitre of a spore suspension of *B. subtilis* 288, added from an automatic pipette. The organism was grown for 10–16 days on a Difco yeast beef agar slope and the growth (mostly spores) washed off and diluted to match Brown's opacity tube no. 2 (Burroughs Wellcome and Co. Ltd). This suspension, which could be stored almost indefinitely, was further diluted 16 times before use. This organism was inhibited by 0·04 μg. micrococcin/ml. The end-point was almost independent of the starting pH within the range pH 6–8.

The end-point was compared with that in a control series containing known concentrations of micrococcin. A sharp end-point was usually obtained with dilution steps differing by 25 %, especially when Evans's peptone was used in the nutrient agar.

When solutions of micrococcin in ethanol or other organic solvents were to be assayed it was necessary to ensure that the concentration of solvent in the first tube was low enough not to interfere. (The titre may be altered by concentrations of solvent well below that which interferes with the growth of the test organism in the absence of micrococcin.) With ethanol, for example, the final concentration must not exceed 1 %. When the solution to be assayed could not be diluted enough to achieve this, a portion was evaporated to dryness, taken up in a few drops of warm ethanol, then made up to volume with water or buffer. One advantage of the fact, made use of in Su's method, that micrococcin is sufficiently stable to withstand autoclaving, is that for much of the setting-up of the test an aseptic technique is not necessary. On the other hand, bulk inoculation is ruled out.

Solid medium dilution methods with other organisms and other end-points, such as the change in colour of a pH or oxidation-reduction indicator, have none of them proved as satisfactory as the method outlined above. However, the reduction of methylene blue in a milk medium, as worked out for other antibiotics (e.g. Reid & Brewer, 1946; Hirsch, 1950) gives results with micrococcin, using *Strep. cremoris* as test organism, within about 2 hr. In some cases the speed with which results are obtained may offset the relative broadness of the end-point.

In an attempt to simplify the dilution procedure, known volumes of serial dilutions were placed in holes in a seeded plate which was incubated. The end-point, taken as the highest dilution which just failed to give perceptible inhibition, was much less sharp than that by Su's method; this modification was therefore abandoned. The same principle had been tried by Su (unpublished), using paper disks or perforated paper disks. It had been hoped that with the latter there would be a sharp all-or-none growth on the surface of the agar exposed by the perforation, since the antibiotic would be diffusing to it in a convergent fashion. Here, too, the results were disappointing.

Lag phase method. This method, developed for the assay of nisin by Hirsch (1950), was tested with micrococcin. The lag phase of *Staphylococcus aureus* and *Strep. faecalis* was prolonged, and with the latter organism a linear relationship occurred between pH and log of micrococcin concentration over the range 0·03–0·1 μg./ml. This range was felt to be too narrow and the method was not pursued.

Turbidimetric method. A few trial experiments gave promising results. The medium consisted of: Evans's peptone 1 %, Marmite 0·15 %, Lemco 0·3 %, NaCl 0·35 %, and glucose 0·1 %, adjusted to pH 7·4 before sterilizing. Three strains of staphylococci were tested, strain 209P being selected as the most suitable. A range of dilutions of micrococcin in sterile water, each 2 ml. in volume in a screw-capped phial, was prepared. To each phial was added 18 ml. of the above medium inoculated with one-twentieth of its volume of a 16 hr. broth culture of the staphylococcus. After 4½ hr. at 37° the turbidities were measured by a Spekker absorptiometer. Typical results are shown in Fig. 1.

The effect of varying some of the conditions (e.g. time of incubation, initial pH value) was next examined. The effect of different incubation times is shown in Fig. 2. When a smaller inoculum was used similar results were obtained, but the turbidity at the end of 4½ hr. was somewhat less. Decreasing the glucose concentration to 0·05 % instead of 0·1 % also diminished the turbidity. It seems likely that satisfactory results could be obtained under a variety of conditions provided an alteration in one factor were balanced by appropriate adjustment of the others. Although this method was not used (since the Su modification was running smoothly) it would seem to have distinct possibilities; results are obtained in a few hours, the method is sensitive, and aseptic conditions are relatively unimportant.

THE DIFFUSION ASSAY OF MICROCOCCIN IN THE PRESENCE OF DETERGENTS

The solubilizing effect of bile and several synthetic detergents on micrococcin (Heatley, Gowans, Florey & Sanders, 1952) suggested the possibility of assaying such 'solutions' by a diffusion method. Detergents have been used in the diffusion assay of polymixin (Stansly & Schlosser, 1947) and nisin (Friedmann & Beach, 1951), but the intention in those cases seems to have been to facilitate diffusion rather than to increase solubility.

Preliminary experiments were made with Triton WR-1339 (a product of Rohm and Haas Co., kindly supplied by Messrs Charles Lennig and Co. [Great Britain] Ltd., 18–20 York Buildings, Adelphi, London, W.C. 2), the non-ionic detergent used by Heatley *et al.* (1952). Even high concentrations did not interfere with the growth of the test organisms. However, it profoundly depressed the antibacterial activity of micrococcin; thus, the minimum inhibitory concentration of the latter to *B. subtilis* was increased 30-fold by 0·1 % Triton and more than 125-fold by 0·9 % Triton. As a result, one effect of the detergent in diffusion assays with very low concentrations of micrococcin was to decrease the size of the inhibition zones. The second effect, resulting from the solubilization of the micrococcin, was to permit, with higher concentrations of the antibiotic, zones larger than the small maximum size obtained in its absence. These points are illustrated in Fig. 3, which shows that as the detergent concentration was increased, the plot of zone diameter against log concentration became linear over a longer range; on the other hand, the higher the concentration of detergent, the smaller was the zone for any given micrococcin concentration (though the decrease in zone diameter was much less than might be expected from the results of dilution tests). When

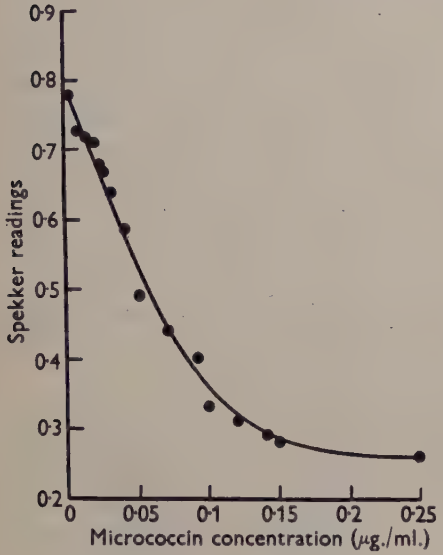

Fig. 1

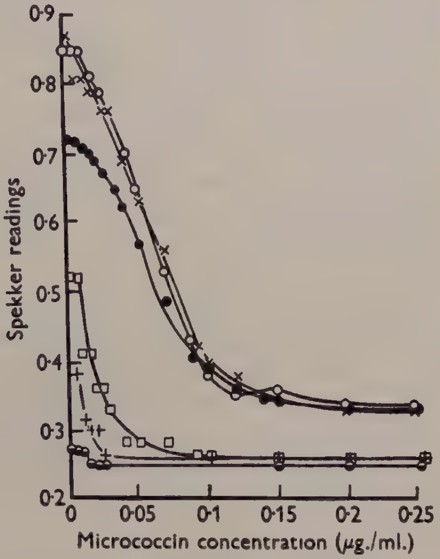

Fig. 2

Fig. 1. The effect of micrococcin concentration on growth (measured turbidimetrically) of *Staph. aureus* 209 P after 4½ hr. incubation at 37°.

Fig. 2. The effect of micrococcin concentration on growth (measured turbidimetrically) of *Staph. aureus* 209 P incubated at 37° for varying lengths of time: ◒——◒, 1 hr.; +——+, 2 hr.; □——□, 3 hr.; ●——●, 4 hr.; ×——×, 5 hr.; ○——○, 6 hr.

detergent was incorporated in the solid medium instead of, or as well as, in the solution in the cylinder, the diminution of the zone size was accentuated; for the main part of the work, therefore, detergent was added only to the solutions to be assayed. Besides Triton WR-1339 (and Triton A-20, which is a solution of it) the following detergents solubilized micrococcin: Crill 10, Tween 80, Teepol, Gemex-X, Aerosol OT, and Tergitol. The last three were strongly inhibitory to *B. subtilis* (the routine test organism) and were not used further.

Degree of curvature and slope of response curves under different conditions. After preliminary experiments the selected detergents were tested in varying concentrations with three concentrations of micrococcin, usually 200, 40 and 8 μg./ml., or half these concentrations, in all possible combinations. The mean zone diameter of eight replicates on fifteen large plates (prepared as described below) in an incomplete block design was used to calculate slope and degree of curvature.

Table 1. *The degree of curvature and slopes obtained for solutions of micrococcin in different concentrations of various detergents*

Detergent	Concentration in phosphate buffer (pH 8·0) (%)	Degree of curvature	Slope
Tween 80	0	+1·6	1·68
	0·5	−1·01	21·95
	1·0	−2·84	23·24
	1·5	−16·38	38·28
	2·0	−13·48	35·84
Crill 10	0·6	−3·81	18·57
	0·8	+0·23	19·93
	1·0	+0·61	21·05
	1·2	+0·09	21·59
	1·4	+0·14	22·12
Triton WR-1339	1·0	−1·48	18·96
	1·05	−2·18	19·50
	1·1	−1·76	19·50
	1·15	−1·4	19·64
	1·2	−1·52	19·66
Teepol	1	−6·61	14·29
	2	−1·63	15·63
	4	+1·08	16·94

The degree of curvature was taken as the sum of the mean responses for the high and low micrococcin concentrations minus twice the mean of that for the middle concentration. Slope was calculated from the mean responses for the high and low concentrations.

The results, summarized in Table 1, show that there is little to choose between the detergents. However, Tween 80 produced readable zones with lower concentrations of micrococcin than did the other detergents, so this compound, at a final concentration of 0·5 %, was selected for routine use.

Linearity tests. With the selected concentrations of detergents eight concentrations of micrococcin ranging from 200 to 7·5 μg./ml. or from 100 to 4 μg./ml. were set up to determine whether the log concentration-zone diameter plot was linear. In every case significant second degree curvature was found, but no higher degree terms were significant. It has been shown (Gridgeman, 1943; Wood, 1944) that when a four-point assay technique is employed, second degree curvature does not affect the estimate of potency ratio.

Routine procedure

Preparation of seeded plates. The preparation of the large assay plates was as described by Brownlee *et al.* (1948), except that each 110 ml. of melted medium was inoculated with 1 ml. of a suspension (diluted to match Brown's opacity tube no. 1) in distilled water, of a 10–14 day slant of *B. subtilis* 288.

Preparation of standard and unknown solutions. The final concentration of Tween 80 was in all cases 0·5 %. The two standard solutions contained 20 and 4μg./ml. and were prepared by diluting with buffer (and Tween) a stock ethanolic solution containing 2 mg. micrococcin/ml. The unknowns were diluted to about the same strength, the low concentration of unknown being exactly one-fifth that in the high concentration. It was felt to be an advantage to leave the first dilutions containing Tween at room temperature for some time, for solubilization of micrococcin to occur, before making the final dilutions. Ethanolic solutions containing less than 2 mg. micrococcin/ml. or solutions in other solvents were evaporated to dryness in a current of warm air, taken up in a few drops of ethanol, then diluted with the appropriate volume of 0·5 % Tween.

Temperature of incubation, etc. The plates were incubated overnight at 32°. They were read in the projection apparatus of Brownlee *et al.* (1948).

Reproducibility. Some indication of this is given by the results of two sets of assays of the standard against itself:

(1) The ethanolic standard solution was diluted with Tween 80 solution to give a final concentration of 20μg. micrococcin/ml. in a Tween concentration of 0·5 %. Nine assays of this solution gave a mean apparent content of 19·53μg./ml., with a standard deviation of 1·50.

(2) The ethanolic standard solution was diluted 1/100 with ethanol. A portion of this was evaporated to dryness and taken up in 0·5 % Tween as described above, to give a solution containing 20μg. micrococcin/ml. Nine assays of this solution gave a mean apparent content of 21·04μg./ml., with a standard deviation of 0·43.

The increment of zone size is about 1·4 mm. for each doubling of concentration, compared with 1·9 mm. for penicillin or 1·7 mm. for streptomycin, under similar conditions.

Assay of crude culture fluid

Untreated crude culture fluids of the micrococcus gave much lower titres of micrococcin by the above diffusion method than by Su's dilution method. Simple autoclaving did not correct this, nor did ethanolic extraction of the acid precipitate of the raw broth. However, diffusion assay of an ethanolic extract of the acid precipitate from autoclaved broth gave titres approaching those found for the untreated broth by Su's method. Evidently the micrococcin occurs in crude broth in some complex or bound form which does not behave in a diffusion assay in the same way as the extracted material. Clearly the applicability of the diffusion method to crude broth cannot be tested by the usual way of observing the recovery of known amounts of added micrococcin. On the other hand, the yardstick by which this can most safely be tested (the dilution method) is itself very inaccurate. In view of the fact that the need for many broth assays was, at any rate temporarily, past, work on these questions was not pursued, but the result of parallel estimations by the two methods on sixteen different samples of culture fluid is given in Table 2.

Table 2. *Parallel estimations on sixteen samples of crude micrococcin-containing broth. (a) By diffusion assay, after ethanol extraction of the acid precipitate of autoclaved samples, (b) by dilution method on solid medium*

	Apparent micrococcin concentration (μg./ml.) when assayed by	
Sample no.	**Diffusion method**	**Dilution method**
1	15	20–28
2	25	36–44
3	37	44–52
4	14	12–20
5	51	52–60
6	19	20–28
7	38	44–52
8	14	20–28
9	48	44–52
10	14	20–28
11	38	44–52
12	50	52–60
13	64	60–68
14	36	36–44
15	54	60–68
16	51	60–68

There remains the question whether the diffusion method is superior to the dilution method for broth assay, in view of the autoclaving, precipitation, washing, etc., which are required. The answer is likely to depend on the personal preferences of the assayist, and also on whether assay plates are in routine use for other purposes and readily available. We feel that there is little to choose between the two methods as regards time consumed, though it is an advantage to have a definite value (by the diffusion method) even though subject to large error, rather than a range of values, as for the dilution method. On the other hand, it must be admitted that the titre given by a dilution method is more fundamental and entails fewer assumptions. Since diffusion methods of assay are purely empirical they should be used with reservation for little known substances, or—as in the present case, perhaps—where the antibiotic may exist in a complex or bound form.

PHYSICAL METHODS OF ESTIMATION

Adsorption on alumina

When a solution of micrococcin in chloroform free from ethanol is passed through a column of Brockmann alumina, the purple fluorescent zone where the micrococcin is adsorbed shows virtually no development when large volumes of fresh ethanol-free chloroform are passed through the column. Thus the volume of the fluorescent zone gives a semi-quantitative measure of the amount of micrococcin present, which is usually 5–15 mg./ml. of loosely packed alumina. This was not investigated further, but might provide the basis for an assay method similar to that described for aureomycin by Seed & Wilson (1949).

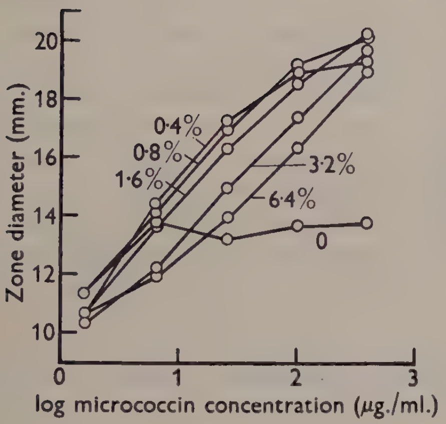

Fig. 3

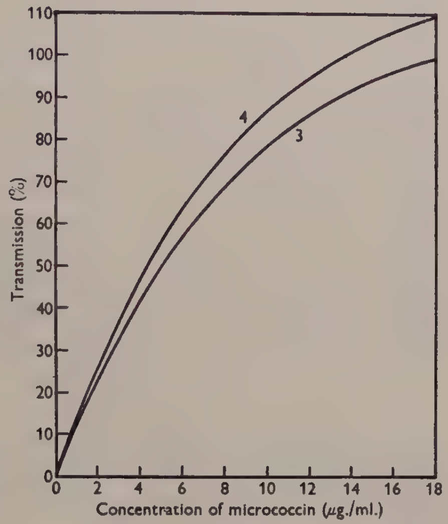

Fig. 4

Fig. 3. **The effect on zone diameter of different concentrations of Triton WR-1339 in the diffusion assay (cylinder plate) of micrococcin. Each point is the mean of four zone measurements. Plates bulk-seeded with *Staph. aureus* NCTC no. 6571. Concentration of Triton as indicated.**

Fig. 4. **Typical calibration curves relating concentration of micrococcin in ethanol to '% transmission', as measured on the Beckman model DU spectrophotometer with fluorimeter attachment. Corex cells, 10 mm. Unknowns in position 3 (lower curve) or position 4 (upper curve) of cell holder.**

Fluorimetric estimation

Micrococcin solutions in ethanol have a powerful purple fluorescence in ultraviolet light. This had early been considered as a method of estimation but was abandoned when a sample submitted for examination in a photographic spectrofluorimeter was reported to have a scarcely detectable fluorescence. However, it was later found that the fluorescence of ethanolic solutions of micrococcin containing 1–15 μg./ml. could be easily and accurately measured in the Beckman spectrophotometer, model DU, fitted with the fluorimeter attachment.

It was customary to use plain ethanol in cell 1, a standard micrococcin solution containing 5μg./ml. in cell 2, and the unknowns in cells 3 and 4. The sensitivity was adjusted so that the standard solution gave a '% transmission' of 50, and the concentration of micrococcin in the unknowns was read off from standard curves (Fig. 4) relating the concentration of micrococcin to '% transmission'. Certain small points, probably well known to users of the Beckman instrument, may be mentioned, as they affected the accuracy of the determinations. (1) The calibration curves for positions 3 and 4 of the cell holder were distinctly different; this was not related to the *cell*, but to the *position* (Fig. 4). (2) The cell holder could be moved back and forth within the carrier a few tenths of a millimetre, its actual position determining the value obtained. Attempts to eliminate the play by springs, packing, etc., were not satisfactory, but errors were avoided by always pushing the cell holder against one end of the carrier and moving the latter very cautiously so as not to displace the former. (3) Since the '% transmission' approached a maximum with concentrations of micrococcin of the order of 30μg./ml. and thereafter declined (due to increasing absorption of light by parts of the solution remote from the photocell), a low reading was not necessarily associated with a low concentration of micrococcin. This could be checked at once by visual examination of the solution in ultraviolet light, or by diluting with a little ethanol and observing whether the reading was thereby increased or decreased.

The intensity of fluorescence of any solution is known to vary with several factors, and the fluorimetric estimation of micrococcin has been purely empirical. The fluorescence was not affected by up to 10 % of chloroform, by changes in the concentration of dissolved oxygen, or by acid, though it was much diminished by alkali. Certain tissue extracts, especially hot ethanolic extracts of blood and dilute haemin solutions, had a marked quenching effect. The effect of water was progressively to enhance the fluorescence up to about 60 % of water, beyond which the fluorescence decreased, eventually almost to zero. The magnitude of the effect depended also on the micrococcin concentration (Heatley & Doery, 1951).

Assay of crude broth by fluorimetry. Ethanolic extracts of broth or other media used for the production of micrococcin (Kelly, Miller & Hale, 1952) all have a fluorescence equivalent to many times the amount of micrococcin likely to be present; moreover, this non-specific fluorescence may well change during the course of the fermentation, making blank corrections impossible. However, the micrococcin can be freed from much of the non-specific fluorescence by acidifying to pH 2, centrifuging and washing the precipitate with water or dilute acid, suspending it in a few drops of water, adding ethanol to dissolve the micrococcin, and removing residual suspended material by centrifuging. Alternatively, the acid precipitate may be collected on a Hirsch micro funnel, washed *in situ* with water or dilute acid, and the micrococcin extracted by passing a measured volume of ethanol through the funnel. The accuracy of the estimation is, however, not great because variable amounts of non-specific fluorescent material are still present (depending, among other things, on the bulk of the precipitate, which varies greatly), and small amounts of micrococcin are lost in the washing. Several experiments on the estimation of micrococcin in broth by preferential extraction into chloroform gave disappointing results.

The specificity of the fluorimetric assay could probably be increased by restricting the spectral range of the incident light or by measuring the fluorescence over a narrow band of wavelengths, but these proposals could not be tested with the apparatus available.

Assay of tissue extracts by fluorimetry. The natural fluorescence of ethanolic extracts of tissues is much less than that of extracts of culture fluids. When tissue extracts contain high concentrations of micrococcin they can be so much diluted that the fluorescence gives a substantially correct value for micrococcin present. When considerable dilution is not possible, errors arise from two sources (apart from high values obtained when much water is present): (1) fluorescence due to substances other than micrococcin; (2) quenching of the micrococcin fluorescence. In most cases a good correction can be applied for both kinds of error by measuring the fluorescence of similar extracts made from tissues of control animals, alone, and with known concentrations of micrococcin added. It is fortunate that the photo-cell used for measuring the purple fluorescence is not very sensitive to the greenish yellow fluorescence of some, but not all, extracts of intestine, liver and kidney.

Estimation by ultraviolet absorption

Micrococcin in ethanolic solution has a broad absorption in the ultraviolet with a peak at 345 mμ., at which Beer's Law is obeyed and $E^{1\%}_{1\,\mathrm{cm.}} = 177{\cdot}5$. This affords an excellent method for estimating micrococcin; there is no need for a standard and, unlike the fluorescence, the absorption is not changed by other components of the solution. However, the latter may themselves absorb; extracts of broth, for example, do so strongly. For tissue extracts corrections may usually be applied by subtracting the absorption of similar extracts from control animals, but unfortunately extracts prepared in an identical fashion from the same organ of two similar animals sometimes show rather wide differences in absorption.

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Culture Media for Large-scale Production of Micrococcin

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SUMMARY: A number of media have been developed for the production of micrococcin. A satisfactory medium consists of corn steep liquor to 1800 mg. N/l. with an aqueous extract of dried autolysed yeast to 1800 mg. N/l., which gives titres of the order of 40–60 μg. micrococcin/ml. in 3 days. A soluble yeast extract such as Marmite may be used in place of the dried autolysed yeast. Moderate aeration is required for good titres but initial pH is relatively unimportant, any value in the range 6·0–7·0 being suitable. The main energy source in sugar-free media is probably the aerobic oxidative deamination of amino-acids. An inoculum that has been pre-incubated gives better results than an inoculum used immediately after it has been prepared. The addition of various inorganic ions to the medium was tried without any detectable influence on titre.

The production of micrococcin (Su, 1948) in quantity was undertaken to provide material with which other workers could examine extraction of the active substance and its behaviour both *in vitro* and *in vivo*. The principal object of the present experiments was to reduce the cost of the medium and to supply information necessary for fairly large-scale fermentation. Although a number of *ad hoc* experiments were carried out in shake flasks, stirred aerated flasks were used for most of the work. The correlation between the results obtained in stirred aerated flasks and those obtained in a 100 gal. stirred aerated fermenter was good.

METHODS

Stirred aerated flasks. The stirred aerated flask (Fig. 1), designed to study general fermentation problems, has been in use for more than 3 years. During this period a number of fungi have been examined. No difficulties, apart from control of foaming, were found in getting a sufficient supply of oxygen to the fungi examined, some of which had an extremely high oxygen demand.

The basis of the design is a bolt-head Chance Hysil flask of 5 l. capacity, normally containing 4 l. of medium. The flask is fitted with a black rubber bung having five moulded holes through which pass the aerators, stirrer, sampling arm and the air outlet filter. The rubber bung is retained in the flask by a suitably perforated metal plate clamped to a metal ring round the neck of the flask by butterfly nuts. The aerator arms, which have a bore of ¼ in., are not restricted at the nozzles. They carry baffle plates preventing vortices and increasing the agitation effect. A relatively small stirrer facilitates assembly. It operates above the aerator outlets and disperses the air in the medium. A range of stirring speeds from 100 to 1000 r.p.m. is available. Greater agitation can be obtained by the addition of links of stainless steel chain to the blades of the stirrer, but excessive speeds with such chains have been found by Mrs Gill-Carey (private communication) to damage extensively a species of fungus. The stirrer shaft operates through a plain metal bearing which is filled with phenolated vaseline immediately after sterilization. A grease-cup, mounted on the shaft below the bearing, prevents the lubricant entering the medium.

Sterile air, pre-heated to the temperature required and automatically humidified, is passed into the flask via a Y-piece, and leaves by the outlet air filter, where its rate of flow is measured on a U-tube flowmeter. Units of four flasks, with an air filter and distributing manifold, are mounted on an aluminium frame which is sterilized with all connexions attached except the drive to the stirrer shafts and those to the air flowmeters. Four such frames are mounted, after sterilization, on a metal rack which carries the flowmeters and an electric motor with pulleys, gears and shafting for stirring. The stirrer shafts are connected by flexible metal cables to the vertical driving shafts. The method of transmitting the drive from the motor can be seen in the photograph of the rack (Pl. 1). The racks run on the monorail system devised by Green (1946) for the penicillin production unit, from which the racks were modified.

Shake flasks. Conical flasks (Pyrex, 200 ml.) containing 50 ml. medium were used on a horizontal shaker giving 84 oscillations/min., the length of throw being 7·5 cm.

Aeration characteristics of the apparatus employed. A number of determinations of the 'aeration constant' (Wise, 1951) for the stirred flasks under conditions normally used were carried out by Mr W. S. Wise. From the results and from experiments on the effect of stirring and aeration, it was clear that the aeration constant necessary to satisfy the peak oxygen demand of the micrococcus used was less than 1·0.

Organism. A strain of *Micrococcus* sp. isolated by Su (1948) was used. This strain is now available from the National Collection of Type Cultures (NCTC 7128).

Assay. The method used to assay fermentation samples was the solid medium dilution method of Su (1948), as modified by Heatley, Kelly & Smith (1952). It is a time-consuming technique, and the number of assay samples was kept to a minimum by pooling equal volumes of samples from replicate fermentations *before* assay. Arbitrary times for sampling were selected after preliminary observations on the course of the development of antibiotic activity.

Conditions used in comparison of media. Fermentations were carried out at 30° and the time of incubation was 4 days. Each flask was stirred at 300 r.p.m. and aerated at 3 l. air/min./4 l. medium. The inoculum was 15 ml. of a 17 hr. stirred aerated culture of the micrococcus grown in nutrient broth at 30°.

Foaming was controlled by the addition of a mixture of D.C. Antifoam 'A' (a Dow Corning silicone product) and tri-amyl citrate. Usual concentrations used were 40 p.p.m. of the former and 100 p.p.m. of the latter, but these were increased when necessary, e.g. with corn steep liquor (CSL), blood preparations and casein. No toxicity or diminution in titre was demonstrated when using large amounts of these agents. The antifoam was added as a solution in chloroform before sterilization.

MEDIA

Su (1948) employed a medium comprising 1 % Lab-Lemco, 1 % Eupeptone no. 2 (Allen and Hanburys, Ltd.), and 0·5 % sodium chloride. It was desirable to find a cheaper and more easily available medium. To this end the materials included in Table 1 were chosen for examination as substitutes for the components of Su's medium. Control runs using Su's medium were always included as a standard of reference for efficiency in micrococcin production. In the first instance, substitutions were based on equal nitrogen contents, but promising combinations were further examined by varying the total nitrogen content and the relative proportions of the two constituents.

Table 1. *Source of supply and total nitrogen (TN) of materials tested in media*

(Total nitrogen determinations by micro-Kjeldahl.)

Material	Source of supply	Total nitrogen (%, w/w)
Constituents of Su's medium		
Lab-Lemco	Oxo Ltd.	6.98
Eupeptone no. 2	Allen and Hanburys Ltd.	15·0
Substitutes for constituents of Su's medium		
Corn Steep liquor (CSL)	Brown and Polson Ltd. (via The Distillers Co. Ltd.)	2·60
Dried autolysed yeast (DAY)*	The Distillers Co. Ltd.	7·16
Brewer's yeast	George's Brewery Ltd., Bristol	2·30
Baker's yeast	The Distillers Co. Ltd.	2·40
Guinness yeast extract	Arthur Guinness, Son & Co. Ltd.	5·60
Yeastrel	Brewers' Food Supply Co.	5·88
Marmite	The Marmite Food Extract Co.	6·04
Meat extract (Argentine)	Armour and Company Ltd.	8·45
Meat meal	British Glues and Chemicals Ltd.	9·15
Maggi's Bouillon powder	Marber and Co. (Food Products) Ltd.	4·80
Bacto-Peptone	Difco Laboratories Inc.	15·5
Fish meal	Hull Fish Meal and Oil Co.	13·2
Casein	Casein (Industries) Ltd.	12·7
Soya flour	Brodie and Co. Ltd.	7·3
Rothes pot ale (concentrated)	The Distillers Co. Ltd.	15·0

* Dried autolysed yeast (DAY) extract was made by autoclaving an aqueous suspension (12 kg. DAY/100 l. water) for a short while and then clarifying with a Sharples centrifuge. Other partially soluble materials were acid-hydrolysed before use.

Finally selected medium based on statistical evaluation of the results of test runs. After numerous experiments in stirred or shaken flasks the best medium was found to be CSL to provide 1800 mg.N/l., DAY extract to provide 1800 mg.N/l., and 0·5 % sodium chloride. Marmite to the same nitrogen content could replace the DAY extract with only slight decrease of titre and had the advantage of eliminating the DAY extraction step in medium preparation. Some batches of CSL could be employed alone at 3600 mg. N/l. but others appeared to contain something which was detrimental to micrococcin production at this concentration. All batches of CSL examined gave satisfactory results in combination with DAY extract or Marmite.

Larger scale production. The process resulting from the work here reported was successfully applied to the production of micrococcin in a 100 gal. stainless steel fermenter which was developed from a Kestner stirred and jacketed vessel. Using CSL/Marmite medium, titres of 40–50 μg. micrococcin/ml. were regularly produced in 66 hr.; under the same conditions in this apparatus Su's medium gave titres of 6–8 μg./ml.

Comments on various factors. The experiments on media composition showed that any of the materials tried were satisfactory substitutes for the peptone in Su's medium, DAY extract and acid-hydrolysed dried blood in particular giving substantially better results. The best substitute for Lab-Lemco was CSL, but even this gave only slightly better results. CSL gave particularly good titres in combination with acid-hydrolysed dried blood, but this was not pursued further as it proved difficult to recover micrococcin from broths which included blood.

The fermentation is relatively insensitive to variations in conditions. The initial pH was varied over the range 6·0–7·4 without detectable effect. It was found to be advantageous to increase the rate of stirring to 600 r.p.m. with an aeration rate of 3 l. air/min., but even under these conditions the aeration constant (Wise, 1951) of the apparatus employed is still less than 1·0.

It was possible to use as inoculum a suspension obtained by washing off 20 oz. slants directly, or after only 7 hr. incubation, with only a slight drop in yield. Increases in concentration of sodium or chloride ion were made without effect on titre, as were additions of potassium, magnesium, phosphate, and sulphate ion. There was some evidence of a slight drop in micrococcin production with very long sterilization times at 15 lb./sq.in. but no difference was observed up to 1 hr.

BIOCHEMICAL OBSERVATIONS

During fermentation in a 100 gal. vessel with CSL/Marmite medium, lactate was rapidly destroyed, acetic acid and ammonia accumulated and the pH value rose (Fig. 2). The ammonia found was equivalent to about 25 % of the available nitrogen.

By the use of cell suspensions of the micrococcus it was observed that α-alanine, glutamic acid and arginine were deaminated with the production of ammonia; histidine, lysine, valine and phenylalanine were not. The deamination of alanine occurred only in presence of oxygen, and the rate of deamination was dependent on the rate of aeration (Fig. 3). This reaction proceeded fastest at about pH 6–6·5, decreasing rapidly on either side of this range. No pyruvate was demonstrated after cell suspensions had been aerated in the presence of alanine even when toluene was present (Stephenson, 1949) but, whether toluene was present or not, acetic acid equivalent to about 15 % of the alanine deaminated was formed. Neither pyruvate, lactate, citrate nor glucose influenced the rate of deamination of alanine by washed cell suspensions. Experiments using gelatine or corn steep protein as substrate indicated that the micrococcus had little proteolytic activity.

Statistical analysis of fermentation experiments in shake flasks showed that alanine could, with advantage, replace part of the CSL nitrogen in CSL/Marmite media, while alanine/Marmite medium was considerably better than peptone/Marmite. Lactate also could replace part of the CSL nitrogen but pyruvate could not (Table 2). Glucose was added in experimental fermenta-

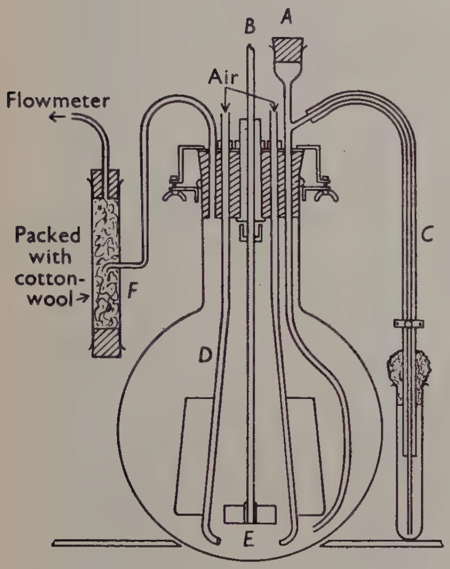

Fig. 1

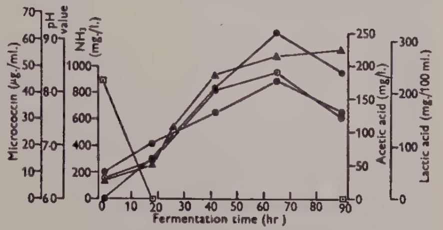

Fig. 2

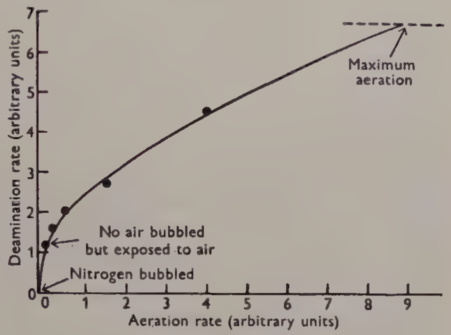

Fig. 3

Fig. 1. Diagram of aerated stirred flask. *A*, inoculator; *B*, stirring shaft; *C*, sampler; *D*, aerator; *E*, stirrer; *F*, outlet air filter.

Fig. 2. Chemical changes in a 70 gal. micrococcin fermentation. Medium: CSL to 1800 mg. N/l.; Marmite to 1800 mg. N/l.; sodium chloride, 0·5 %. Micrococcin, —●—; pH, —△—; ammonia, —⊙—; acetic acid, —⊗—; lactic acid, —⊡—.

Fig. 3. Form of curve relating aeration rate to rate of deamination of α-alanine by the micrococcus.

Table 2. *The influence of alanine, pyruvate, and lactate on micrococcin production*

(*Basal medium*: CSL to provide 1·8 g. N/l.; Marmite to provide 1·8 g. N/l.; Sodium chloride 0·5 %; Sodium sulphate 0·05 %; pH value = 6·5.)

Medium	Micrococcin μg./ml. (mean)
Undiluted	56
Diluted 1/2	28
Diluted 1/2 + alanine to restore to 3·6 g. N/l.	74
Diluted 1/2 + pyruvate to same molarity as alanine above	28
Diluted 1/2 + lactate to same molarity as alanine above	45

tions as an alternative energy source, but it was observed that the low pH which resulted inhibited micrococcin production. The use of calcium carbonate in such fermentations, was not successful as it did not maintain the pH high enough (above pH 6) for maximum production.

Our thanks are due to Prof. Sir Howard Florey, F.R.S., for suggesting this problem and for continued interest in the work; to Miss P. F. Boyd for maintenance of cultures and production of inocula; to Miss N. Smith who was responsible for all assays; to Mr R. C. Codner who suggested modifications of the Kestner vessel; to Mr J. M. Whitmarsh for help in the early stages of this work.

The Engineering Division of The Distillers Co. Ltd., Research and Development Department, kindly assisted in the design and construction of the stirred and aerated fermentation apparatus.

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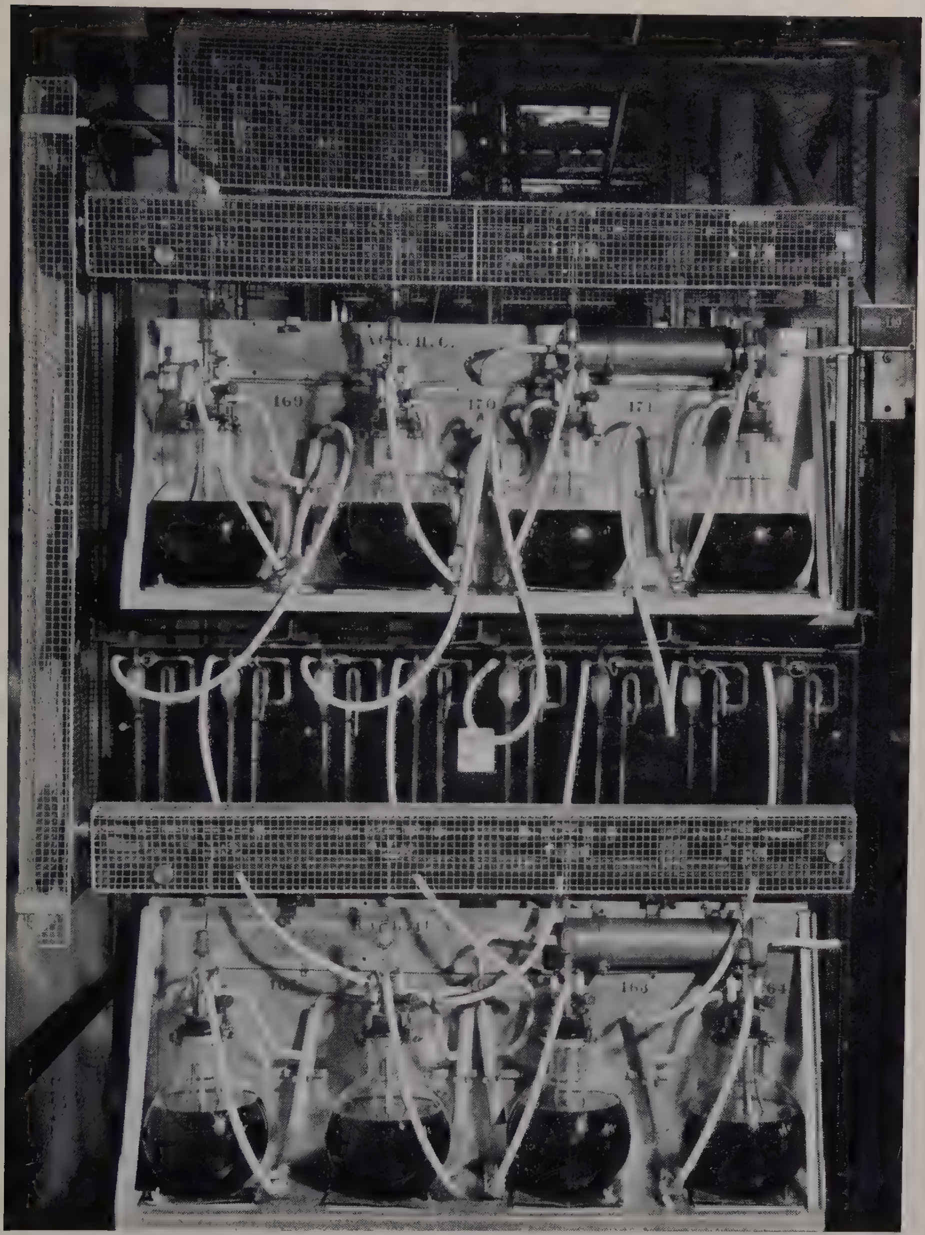
EXPLANATION OF PLATE

Plate 1

Fermentation rack showing two frames mounted on one side with stirrers connected up.

(*Received* 25 *May* 1951)


169
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B. K. Kelly, G. A. Miller & C. W. Hale—Production of micrococcin. Plate 1

Crawford, Kathleen, Heatley, N. G., Boyd, Pamela F., Hale, C. W., Kelly, B. K., Miller, G. A. & Smith, Nancy (1952). *J. gen. Microbiol.* 6, 47–59.

Antibiotic Production by a Species of *Cephalosporium*

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SUMMARY: The antibacterial activity of a strain of a *Cephalosporium* sp. was found to be due to a number of antibiotics. One group of these, named Cephalosporins P, is soluble in organic solvents and active only against Gram-positive bacteria.

The P antibiotic(s) is produced in good yield in deep culture in a medium comprising corn steep liquor and glucose at an initial pH of 6·3. A high aeration rate assisted by adequate stirring is essential. Submerged cultures 72 hr. old form convenient inocula. Under optimum conditions with 2 % (v/v) of such an inoculum the maximum titre is reached after 47–50 hr. at 24°, and thereafter falls.

The production of a diffusible antibacterial substance by a species of *Cephalosporium* was reported by Brotzu (1948) in an apparently little-known paper which was brought to the notice of Sir Howard Florey by Dr C. O. S. Blyth Brooke. Prof. Brotzu kindly provided a culture of the organism, which was one of a number showing antibacterial action which he has isolated from the sea near a sewage outfall off Sardinia. This locality had been chosen with the expectation that antagonistic organisms would be plentiful there in view of the 'self-purification' known to occur in sewage. The organism, *C. acremonium* or a closely related species, grew at room temperature on a number of complex solid and liquid media preferably containing added carbohydrate (e.g. glucose, lactose or starch) and a 'titre' of 1/10 to 1/15 (test organism not specified) was reached in 4–8 days. The addition of various amino-acids, extracts of yeast, corn germ, peas, etc., had little effect on the titre which was, however, about six times greater from one daughter strain selected from hundreds tested. Streaking tests on solid media showed marked inhibition of *Salmonella typhi*, *S. paratyphi B*, *Shigella shiga-kruze*, *Vibrio cholerae*, *Staphylococcus aureus*, *Brucella melitensis*, *Bacillus anthracis* and *Pasteurella pestis*. There was no action against *Pseudomonas aerogenes*. Brotzu found that the active substance was rather unstable and various methods of extraction and purification were tried with little success. An active extract could be made by concentrating the filtered broth (which was usually yellow) to 1/10 its volume in a current of air at 25°, adding 1·5 vol. of 98 % ethanol, removing the precipitate by filtration through cotton-wool, and concentrating the ethanol extract at 37–40°. In an improved process (Brotzu, personal communication to Sir Howard Florey) the ethanolic extract obtained as above was shaken with about 3 vol. of acetone. The precipitate thereby formed was dissolved in a little water, mixed with 5 vol. of 96 % ethanol, then, after discarding the precipitate, with 30 vol. of acetone. The precipitate, dissolved in a small volume of water, was treated with charcoal (Merck, 1 g./10 ml.) for 1 hr. After centrifuging, the supernatant, which was almost colourless, contained a good deal of activity. Both the crude culture-fluid and extracts of it were tested clinically. Good results were claimed from local treatment of pyogenic infections and from the general treatment of typhoid and paratyphoid fevers, and brucellosis.

The work reported here falls into two parts: preliminary observations made at Oxford, and further work relating to large-scale production done at Clevedon. It was established that the fungus could form one or more substances active mainly against Gram-positive organisms, and one substance also active against Gram-negative species. It is probable that the latter was responsible for most of Brotzu's observations. The preliminary work at Oxford reported here must have been mainly concerned with the Gram-positive inhibitory substance(s). The isolation and characterization of the latter are described by Burton & Abraham (1951), and some bacteriological and *in vivo* studies of them are reported by Ritchie, Smith, & Florey (1951).

PRELIMINARY OBSERVATIONS

Assay. Inhibitory material from cultures of the *Cephalosporium* strain diffused readily through agar and was easily assayed by the cylinder-plate method (Heatley, 1944) against either *Staph. aureus* NCTC 6571 or a laboratory strain of *Corynebacterium xerosis*. With either organism there was an increment of 3–4 mm. in zone diameter for each doubling of concentration of the inhibitor. A crude active culture-filtrate was tentatively selected as a standard, being assigned a potency of 1 unit/ml.; other fluids and, later, a dry powder, were standardized in terms of it. Though both solid and fluid preparations were liable to lose activity quickly, there was no evidence that any of the standards (kept at +4°, and, if in powder form, over calcium chloride also) lost appreciable activity. A concentration of 1 unit/ml. gave a zone 17–22 mm. in diameter with either of the above test organisms.

Preparation of inocula. Various solid media, with and without calcium carbonate, were tested for the production of spores by this strain of *Cephalosporium*. Spores were freely formed after 8 days at 24° on a medium (pH 7·4) consisting of 1 % Lemco, 1 % peptone and 1 % glucose. Modifications containing less nitrogen, or simple dilutions of beer or corn steep liquor (CSL), also allowed free sporing, although more slowly. The spores were more readily dispersed when glucose was omitted from the Lemco peptone glucose medium. Aerated cultures also formed very satisfactory inocula.

Cultivation experiments

In static culture. Active material was readily produced in shallow layers (1 cm. or less) of Lemco peptone glucose medium. Up to 2 u./ml. were formed after 10–12 days at 24°, or after 7–10 days at 37°. Up to 5 u./ml. were obtained in the same medium in successive harvests from cellophan sacs (Heatley & Florey, 1946), but these became fragile and usually broke within 1–3 weeks, as did sacs of collodion.

When the active medium in a shallow static culture was replaced by fresh medium, the latter in turn became active in about the same length of time as required for the first growth. However, it was difficult to remove culture fluid without sucking away large amounts of the slimy tenuous mycelium. Attempts were made to overcome this by growing the fungus on cellophan completely covering the surface of fluid medium in trays, but although good titres were obtained the cellophan became brittle and tore easily. The fungus was also grown on a thin layer of a mixture of nutrient agar and cork powder which, it was hoped, would float when fluid medium was subsequently introduced. Cork fragments were used in a similar way by Gill-Carey (1950). Though promising, this scheme was abandoned when good results were obtained from submerged aerated cultures.

In aerated submerged culture. The organism grew well in aerated submerged culture and antibiotic activity was obtained comparable to that formed in stationary culture provided the rate of aeration was not less than 1 vol. air/vol. medium/min. An improved yield was obtained with a medium consisting of 5 % (v/v) CSL + 4 % glucose. With 2·5 % (v/v) CSL + 2 % glucose the same titre was reached, but rather more rapidly (4–5 days); with a still more dilute medium the titre was lower. Titres were substantially the same from cultures grown at 24, 29, or 37°, and inhibitory material from cultures in Lemco peptone glucose medium had much the same range of antibacterial action as that from the CSL glucose medium.

Examination of related species of Cephalosporium. Miss A. Beverwyck of the Centraalbureau voor Schimmelcultures, Baarn, kindly examined the organism and stated that it most nearly resembled *C. acremonium* Cda., *C. album* Preuss, and *C. Ballaggii* Oomen. These however did not produce the yellow colour in the medium characteristic of Brotzu's organism, nor, in tests at Oxford on solid medium, was any antibacterial activity demonstrated.

Physical and chemical properties

When culture fluid was heated to 100° for 10 min. at pH 2, 6·5 and 8·8 the approximate losses of activity were 40, 60, and 65 % respectively. The crude culture fluid did not appreciably lose activity when evaporated to dryness on a heated watch-glass: the active material in such a dried residue was insoluble in hexane, slightly soluble in ether or chloroform, and more soluble in acetone, ethanol, and water.

The active material did not sublime under reduced pressure. It was extracted from a partially purified watery solution into ether at pH 2, 4·8, or 6·5, but not at pH 9·6. A proportion of the yellow pigment passed into ether at pH 2, but not at the other three pH values. Thus extraction into ether at pH 5, followed by re-extraction into water at pH 9·6, appeared to be a useful step in purification.

The antibiotic material was fairly readily adsorbed on Farnell Grade 14 charcoal at pH 2, but it seemed to be necessary to acidify the solution *before* adding the charcoal, otherwise the adsorption was less complete. The adsorbed material was not eluted with neutral or alkaline buffer. Activity was not destroyed when the charcoal was dried, and active material could be eluted with acetone or ethanol or, less effectively, with ether. Elution was almost complete after Soxhlet extraction with ether for 2 hr., but there was evidence of slow destruction on refluxing an ether solution of the material for several hours. The yield after elution from charcoal was in no case greater than 50 %.

Active material was adsorbed from chloroform on to an alumina column and could be eluted from the appropriate zone thereof with ethanol. Small quantities of crystalline material were seen in the residue from one such active fraction, but there was insufficient to ascertain whether the crystals were antibiotic.

EXPERIMENTAL WORK AT CLEVEDON

In the first fermentations carried out at Clevedon the antibiotic titre against *B. subtilis* showed abnormal fluctuations throughout the fermentation, and the amount of activity extracted into amyl acetate (Burton & Abraham, 1952) bore no resemblance to that in the broth; it was also observed that part of the activity was lost under acid conditions. A likely explanation was the production of more than one antibiotic, one at least being insoluble in amyl acetate. The first part of the work reported below describes experiments to determine the number and nature of the inhibitors present in the fermentation broth, and to distinguish between them. A final section then deals with the larger scale production of one group of these inhibitors.

The demonstration and differentiation of various antibiotics in crude Cephalosporium *broth*

Behaviour to preparations containing penicillinase. The 'penicillinase preparations' mostly used were prepared from (1) *B. subtilis* G.B.S. 2 (by courtesy of The Distillers Co. (Biochemicals) Ltd.), and (2) *B. subtilis* NCTC 6346 by the method of Duthie (1944). In general, 1 vol. of the fluid under test was incubated with 0·1 vol. of a suitable dilution of the enzyme preparation in 0·5M phosphate buffer (pH 7·8) at 35° for 2 hr.

It was shown by Burton & Abraham (1952) that penicillinase prepared from *B. subtilis* NCTC 6346 inactivated the antibiotics produced by this *Cephalosporium* strain. However, we found that with the first enzyme preparation mentioned above, only partial inactivation was obtained. Table 1 shows the results with such a preparation acting on samples taken at different time intervals from shake-flask cultures containing CSL (840 mg. N/l.) + 2 % glucose, at initial pH values of 7·0 and 6·4. The results suggest that an acid fermentation favoured the production of an antibiotic insensitive to the enzyme preparation while an alkaline fermentation favoured the production of both types of antibiotic. Later in the fermentation, when alkaline conditions tended in any case to supervene, the distinction was less marked. Similar experiments on other types of fermentation have confirmed this.

Separation by paper chromatography. Samples of culture fluid were spotted onto ½ × 22 in. strips of Whatman no. 1 filter-paper which had previously been soaked in 20 % potassium phosphate (pH 6·15) and dried. They were suspended in a vessel similar to that of Goodall & Levy (1947), and developed for some hours with amyl acetate saturated with water. After drying at room temperature they were placed on the surface of seeded or unseeded nutrient agar for 1 hr., then removed and the agar incubated or streaked with test organisms and then incubated (Pl. 1, figs. 1, 2 and 3).

Table 1. *The partial inactivation of* Cephalosporium *antibiotics by an enzyme preparation containing penicillinase from* B. subtilis *G.B.S.*2

Starting pH	Fermentation time (hr.)	Titre (arbitrary units)	
		Before treatment	After treatment
7·0	48	9·2	4·7
	72	17·2	11·7
	96	8·5	5·2
6·4	48	4·6	3·1
	72	12·9	11·2
	96	5·7	2·1

When treated in this way and tested against *B. subtilis* 288, crude active broth gave two inhibition zones having R_F values (Consden, Gordon & Martin, 1944) of 0 and 0·95–1·00; in broth treated with 'penicillinase' only the lower zone ($R_F=0{\cdot}95$) occurred. When the broth was first held at pH 2 at room temperature for a few hours, all or nearly all the top zone ($R_F=0$) disappeared. On the other hand, in broth extracted at pH 6 with an equal volume of amyl acetate only the top antibiotic zone remained in the broth. The amyl acetate extract showed only one zone ($R_F=0{\cdot}95$) with some broths, though with others a zone at $R_F=0$ also occurred. The material responsible for the latter was not inactivated by 'penicillinase' from *B. subtilis* G.B.S. 2, and differed from the non-extracted top zone antibiotic in other ways. Pl. 1, fig. 4, illustrates chromatograms of material treated in various ways.

Range of antibacterial action. Strips of filter-paper soaked in the solution under test were temporarily applied to a nutrient agar surface which was then cross-streaked with test organisms and incubated. Sometimes portions of chromatographically developed paper strips were cut out and tested in the same way.

The range of antibacterial action of the three types of antibiotic are shown in Table 2. It will be seen from Table 2 that in general the antibiotics soluble in amyl acetate inhibited only the Gram-positive organisms tested, while the acetate-insoluble material inhibited a number of *Salmonella* spp. and *Klebsiella pneumoniae*, but not *Staph. albus* NCTC 7292. Accordingly, the acetate-insoluble antibiotic was called 'N' and the acetate-soluble antibiotics 'P(*a*)' ($R_F=0{\cdot}95$), and 'P(*b*)' ($R_F=0$). Table 3 shows some properties of the various antibiotic substances so far elucidated.

Table 2. *The inhibition of bacterial growth by antibiotics produced by the* Cephalosporium *strain*

	Antibiotic		
	Insoluble in amyl acetate	Soluble in amyl acetate	
Test organism	N*	P(*a*)*	P(*b*)*
B. anthracis (Oxford)	++	++	++
B. megatherium NCTC 2605	++	+	+
B. mesentericus NCTC 6353	++	+++	+++
B. subtilis (Oxford)	++	+	+
B. subtilis Strain 288 of Distillers Co. Ltd.	+++	++	++
Bodenheimer organism (Trussell *et al.* 1947)	0	0	0
Corynebacterium simplex NCTC 4215	0?	++	++
C. xerosis (Oxford)	++	++	++
Bacterium coli (Oxford)	0	0	0
Serratia marcescens NCTC 1377	0	0	0
Klebsiella pneumoniae (Oxford)	+++	0	0
Micrococcus lysodeikticus (Oxford)	++	+	+
Micrococcus sp. NCTC 7218	++	+++	+++
Mycobacterium phlei (Oxford)	+++	+++	+++
Pseudomonas pyocyanea (Oxford)	0	0	0
Salmonella enteritidis (Oxford)	++	0	0
S. typhi (Oxford)	++	0	0
S. typhimurium (Clevedon 6)	++	0	0
Sarcina sp. (Clevedon 8)	++	++	++
Staph. albus NCTC 7292	0	++	++
Staph. albus (Oxford)	0	+	+
Staph. aureus (Clevedon 4)	++	+++	+++
Staph. aureus Strain G of Distillers Co. Ltd.	++	++	++
Staph. aureus Strain 209 P	+	+++	+++
Staph. aureus (Oxford)	++	+	+
Strep. lactis NCTC 662	++	++	++
Strep. pyogenes (Oxford)	++	++	++
Pasteurella sp. (Oxford)	+++	++	++

0 = no inhibition; + = some inhibition; ++ = marked inhibition; +++ = very marked inhibition.

* For explanation see text, p. 51.

Table 3. *Some properties of the antibiotics produced by the* Cephalosporium *strain*

	Antibiotic		
Property	N*	P(*a*)*	P(*b*)*
Antibiotic to *Staph. albus* NCTC 7292	−	+	+
Antibiotic to *Klebsiella pneumoniae*, and many Gram-negative organisms	+	−	−
Extracted into amyl acetate from acid solution	−	+	+†
Inactivated by penicillinase from *B. subtilis* G.B.S. 2	+	−	−
Labile in acid solution	+	−	−
Mainly produced in slightly alkaline fermentations	+	−	?
Mainly produced in slightly acid fermentations	−	+	?

* These symbols have same significance as in Table 2; see p. 51.

† But possibly strongly adsorbed to paper fibres.

Test organisms for differential assay. Staph. albus NCTC 7292 was strongly inhibited by P(*a*) and P(*b*), but not at all by N, while *K. pneumoniae* was strongly inhibited by N but not at all by either P(*a*) or P(*b*). These organisms were therefore tested for differential assay and appeared to be entirely satisfactory.

CULTIVATION OF THE *CEPHALOSPORIUM* STRAIN AND THE PRODUCTION OF THE 'P' ANTIBIOTICS

Culture maintenance and preparation of inocula

A 15-day culture was suspended in ascorbic acid freeze-drying medium (Naylor & Smith, 1946) in double constriction ampoules, frozen at −78°, and dried at low pressure. The ampoules were afterwards sealed at atmospheric pressure and stored at 4°. The percentage survival of the dried material has so far (18 months) proved satisfactory. The mere process of drying was found to have no detectable effect on subsequent antibiotic titre. An ampoule was opened each month and slants sown from it were used for inoculating weekly stock cultures.

Spore inoculum. 'Bowpanel flats' (20 oz.), each containing 110 ml. of the surface culture medium described by LePage & Campbell (1946), with the addition of 2 % agar, were sown with 1·5 ml. of a spore suspension, incubated for 14 days at 24°, and stored for not less than 1 day or more than 6 days at 4°. Glass beads in 40 ml. distilled water were used to wash off spores as required, a thick uniform suspension being obtained.

Inoculum by submerged culture. When large quantities of inoculum were required, submerged cultures were used. They were prepared in 10 l. Pyrex aspirators agitated by a paddle type stirrer running at 150 r.p.m. and aerated by a horizontal perforated glass tube entering through the lower outlet of the aspirator. A second tube from this outlet was attached to a sampling device. Each aspirator was charged with 8 l. of CSL+glucose medium (see below), and autoclaved 1 hr. at 120°. Spore suspension (160 ml.) was added as inoculum, and the aspirators were aerated (with stirring) at 24° for 72 hr. After testing for contamination, the sampling device was replaced by a separately sterilized apparatus consisting of a calibrated measuring device with a hooded pipette, from which uniform volumes of inoculum could be dispensed.

Size of inoculum. In most fermentations the inoculum, of spores or submerged growth, was 2 % (v/v) of the medium to be inoculated.

Fermentation vessels

Shake flasks. Narrow-mouthed 250 ml. Erlenmeyer flasks containing 50 ml. of medium were agitated on a horizontal shaker giving 84 oscillations/min.; length of throw 7·5 cm.

Stirred aerated flasks. The constructional details and use of these 5 l. bolt-head flasks is described by Kelly, Miller & Hale (1952). The flasks contained 4 l. of medium, could be aerated at 6 l./min., and the stirrer (fitted with short lengths of stainless steel chain if desired) could run at 900 r.p.m. Speed of stirring and aeration rate could be varied over a wide range. The flasks were arranged in groups of 16 to facilitate statistical design and analysis of the experiments.

Stirred aerated stainless steel fermenter. A 100 gal. fermenter modified from a stainless steel mixing vessel was employed; stirring was at 400 r.p.m. and aeration at 20–21 cu.ft./min.

Antifoam agents. It was necessary to add antifoam agents to the 5 l. and 100 gal. fermenters. A mixture of tri-amyl citrate (400 p.p.m.), and Dow Corning Silicone 'A' (160 p.p.m.) was usually used to control foaming in the 5 l. vessels; lower concentrations could be used in the 100 gal. fermenter. Certain batches of CSL caused more foaming than others and more antifoam mixture had to be used. No decrease of yield of antibiotics was found when larger concentrations of antifoam were added in controlled experiments.

Assay

The method of assay of Cephalosporin P was similar to that developed for streptomycin by Brownlee *et al.* (1948). At first *B. subtilis* 288 was used as test organism, but was replaced by *Staph. albus* NCTC 7292 when this was shown to be specially inhibited by the P antibiotics.

The medium consisted of: Evans's peptone, 1 %; sodium chloride, 0·35 %; Armour's meat extract, 0·3 %; Yeastrel, 0·15 %; agar, 2 %. The pH was adjusted to 7·4 and the medium autoclaved, filtered, distributed and re-autoclaved at 15 lb./sq.in. for 20 min. Volumes of 110 ml., held at 45°, were inoculated with 1 ml. of a suspension of the test organism, then poured into the special plates. The suspension was prepared from 48 hr. nutrient agar (pH 7) slants of the *Staph. albus*, and was diluted to match opacity four of the Brown's opacity tubes (Burroughs Wellcome and Co. Ltd).

Preparation of samples for assay. The samples of culture fluid were centrifuged and diluted in 0·2 M phosphate buffer (pH 7·0) to give two solutions at *c.* 4 and 2 u./ml. Standard solutions were also prepared to contain exactly 4 and 2 u./ml. Samples of cephalosporin P in amyl or butyl acetate were prepared by evaporating a known volume to dryness in a current of air; the residue was dissolved in 0·1 ml. acetone then made up to volume in pH 7 phosphate buffer.

Procedure. This was as described by Brownlee *et al.* (1948), the plates being incubated for about 16 hr. at 32°, and the zones measured on an enlarger. The method of calculation was that described by Brownlee, Lorraine & Stevens (1949).

Unit of activity, standard, etc. As soon as a solid preparation believed to be substantially pure P antibiotic(s) became available, the unit was redefined as the activity in 10 μg. of this solid preparation. This unit was approximately equivalent to that originally adopted.

RESULTS

Sporulation medium. Cultures on a wide variety of stock media were compared, and from the best of them spore suspensions were prepared and counted in a haemocytometer. LePage & Campbell (1946) medium was found to give an appreciably higher spore count, especially from older slants, and was therefore adopted as a suitable sporulation medium. A very good growth was obtained in 3 days at 24°, and by 14 days complete sporulation had taken place, indicated by the somewhat translucent appearance of the growth. Slants washed off earlier than 14 days and without at least 1 day's storage at 4°, were extremely difficult to suspend; the resulting suspensions then contained large pieces of felt and formed very poor inocula.

Effect of age of sporing culture on antibiotic production. Spore suspensions from slants of 9 days and 3, 4, 6, 8, 9 and 12 weeks were compared for antibiotic production in shake flasks. The 3- and 4-week slants appeared to give the best titres. When slants between 10 and 28 days old were carefully compared, the optimum yield was from slants 14–18 days old, though those up to 28 days old were almost as good.

The effect of repeated subculture on yield of antibiotic. With frequently subcultured strains the antibacterial activity developed more slowly than with seldom transferred strains, although the same titre was reached in each by 72 hr. The number of transfers was therefore kept to a minimum.

Investigations on different inocula

Spore inoculum. Various volumes of spore inoculum, prepared as above, were tested in shake culture flasks. With the inoculum forming 2, 3 or 4 % (v/v) of the volume of medium the same optimum titre was reached. With smaller volumes of inoculum activity appeared later and often did not reach the same level.

Submerged inoculum. Whether in shake flasks or in 10 l. aspirators, 72–96 hr. appeared to be the optimum incubation time for submerged inocula preparation. The inoculum was tested in both shake flasks and in 5 l. stirred aerated flasks; the results from the latter vessels are shown in Table 4.

Table 4. *Influence of age of submerged culture inoculum on Cephalosporin P production*

(The inoculum was grown in 10 l stirred aerated aspirators for the times shown.)

Period of fermentation (hr.)	**Age of inoculum (hr.)**			
	24	**48**	**72**	**96**
	Units of Cephalosporin P/ml. (av. of four fermentations)			
23	•	1·1	2·2	2·4
44·5	0·95	4·9	10·3	10·1
49	1·8	5·9	11·3	9·8

The optimum size of submerged inoculum was investigated in shake flasks and in 5 l. stirred aerated flasks. In both types of vessel maximum production was obtained with a 2 % (v/v) inoculum, though with 4 % (v/v) the same titre was reached more rapidly. With less than 2 % (v/v) inoculum, rate of production and ultimate yield were smaller. Table 5 shows the results of an experiment with the 5 l. flasks.

Table 5. *Influence of size of inoculum on the production of Cephalosporin P*

Period of fermentation (hr.)	Size of inoculum, as % of total volume			
	0·5 %	1 %	2 %	4 %
	Units Cephalosporin P/ml. (av. of eight fermentations)			
24	0·7	1·6	2·9	5·2
28	1·6	3·2	4·9	8·2
47	9·7	10·5	11·2	13·0
50	12·1	12·0	13·1	13·0

Comparison of spore and submerged inoculum. Table 6 shows the titres obtained in shake flask cultures inoculated with 2 % (v/v) of (*a*) spore inoculum, or (*b*) 72 hr. submerged inoculum. The true peak titres of the latter may well have been missed, but no significant difference in level is suggested.

Table 6. *Comparison of spore and submerged culture inocula*

Type of inoculum	Period of fermentation (hr.)		
	40	64	88
	Units Cephalosporin P/ml. (av. of four fermentations)		
2 % (v/v) spore suspension	4·2	7·6	11·0
2 % (v/v) submerged culture	6·4	9·8	9·1

Composition of medium and fermentation conditions

Corn steep liquor + glucose medium. A number of experiments were set up in stirred aerated 5 l. flasks and aspirator bottles with various concentrations of CSL (1–5 %, v/v), and glucose (1–4 %, w/v). These all indicated as optimum 2·5 % (v/v) CSL + 2 % (w/v) glucose. Owing to variations in Kjeldahl nitrogen between different batches of CSL, in later work the amount of CSL taken was that required to provide 840 mg. N/l., rather than 2·5 % (v/v).

Inorganic additions to basal medium. Various concentrations of sodium, potassium, iron, magnesium, chloride, sulphate and phosphate were added as individual salts and in combinations. No increase in P antibiotic was obtained, although some salts depressed both growth and titre.

Other media. None of the other media tested gave higher yields of the P antibiotics than the above basal medium. In view of the low cost and simplicity of the latter more exhaustive investigations were not made.

Temperature of fermentation. The temperature adopted for all fermentations was 24°. At 28° no change in titre was observed, so that the slight rise of temperature in the 5 l. flasks which began after 20 hr. and reached its maximum of 3° after 50 hr. was probably of no significance.

Aeration and stirring. The air demand of this *Cephalosporium* strain seems to be far higher than that of other fungi grown under similar conditions, and high stirring and aeration rates were required. This is illustrated in Table 7, and by the fact that full antibiotic production took longer in shake flasks than in well aerated and stirred flasks.

Hydrogen-ion concentration. The pH value of the medium had a marked effect on the nature of the antibiotics formed. For the production of P antibiotics, an initial pH of 6·3 (after sterilization) was adopted as standard.

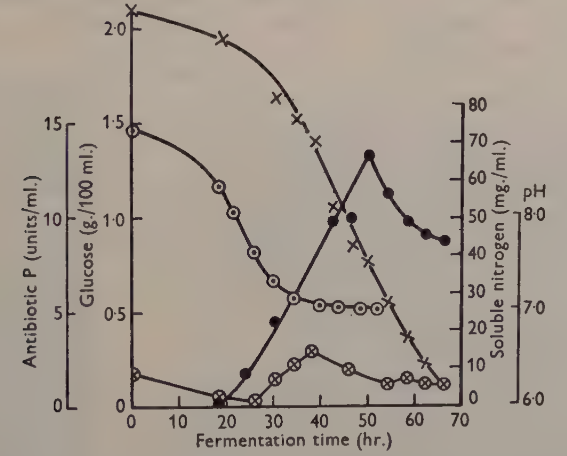
Table 7. *Influence of stirring and aeration on Cephalosporin P production*

Aeration rate (l./min.)	Stirring rate (r.p.m.)	Period of fermentation (hr.) 24	 26	 30	 48	 50
		Units Cephalosporin P/ml.*				
12	900	2·6	3·4	6·8	13·0	14·0
6	900	2·4	4·8	8·5	13·0	13·0
12	600	1·3	1·6	2·9	7·1	7·7
6	600	0·9	1·5	2·1	4·6	4·8

* Av. of fifteen fermentations obtained by bulking samples.

Fermentation in the 70 *gal. fermenter*

With the standard medium the optimum period of fermentation at 24° was 47–50 hr., when the titre was 9–13 u./ml. Fig. 1 shows the course of antibiotic production and some chemical changes in the medium. It will be seen that the titre falls off rapidly after the peak has been passed. After growth the 70 gal. of brew were adjusted to pH 5·0 and the mycelium removed by filtration through a cloth bag-filter. The filtrate was extracted with butyl acetate at pH 6–6·5 by the methods described by Burton & Abraham (1951), practically 100 % of the P antibiotics being so extracted.



Text-fig. 1. Changes occurring during a 70 gal. Cephalosporin P fermentation. ●——●, Cephalosporin P; ×——×, glucose; ⊙——⊙, soluble nitrogen; ⊗——⊗, pH. In this particular fermentation, the initial glucose concentration was increased to 3 % of the impure monohydrate.

Our thanks are due to Sir Howard Florey for suggesting this work and for his interest and encouragement.

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EXPLANATION OF PLATE

Fig. 1. Examination of antibiotics produced by a *Cephalosporium* sp., by bacterial spectra on zones from chromatographically developed filter strips placed on agar seeded with *B. subtilis* 288. Top N zones and lower P zones were streaked with various test organisms. The order of streaking is varied on the two strips so that each organism may cross each zone once. Test organisms: (1) *K. pneumoniae* (Oxford); (2) *Staph. aureus G*; (3) *Staph. albus* NCTC 7292.

Fig. 2. Zones from paper strip on agar plate seeded with *Klebsiella pneumoniae* (cf. Fig. 1). Showing top zone, Cephalosporin N. Presence of lower zone (Cephalosporin P antibiotics) demonstrated by inhibition of certain streaks. Test organisms: (1) *Staph. aureus* (Oxford); (2) *S. typhimurium* (Clevedon 6); (3) *Staph. albus* NCTC 7292; (4) *B. subtilis* (Oxford).

Fig. 3. Zones from paper strip on agar plate seeded with *Staph. albus* NCTC 7292 (cf. Fig. 1). Showing lower zone, Cephalosporin P. Presence of top zone (Cephalosporin N) demonstrated by inhibition of streaks. Test organisms: (1) *K. pneumoniae*; (2) *S. typhimurium*; (3) *Staph. aureus*.

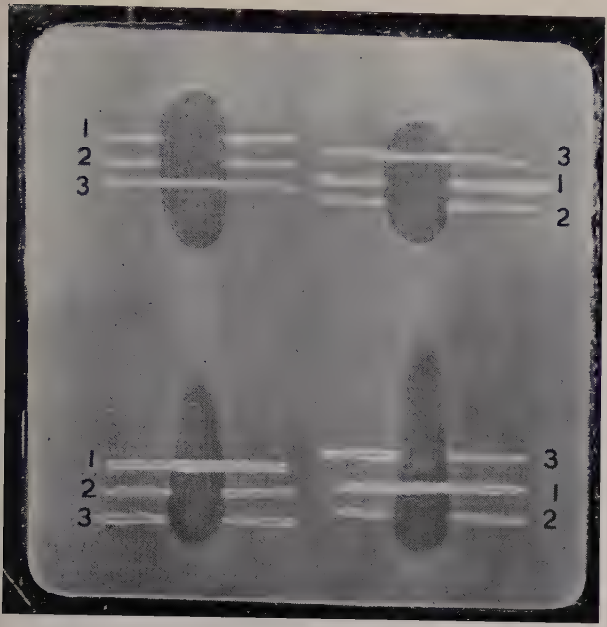
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Fig. 1

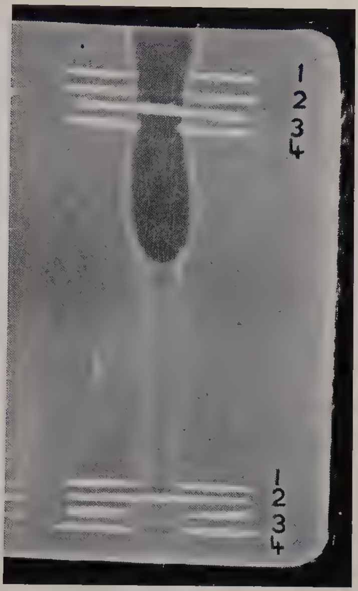

Fig. 2

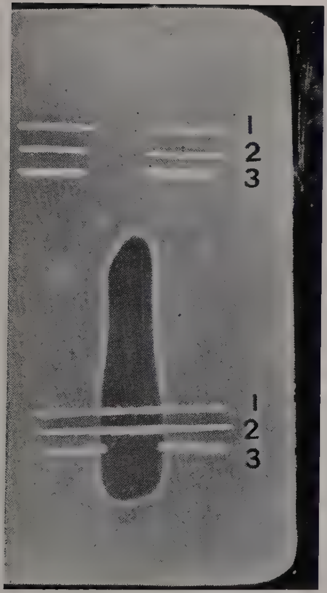

Fig. 3

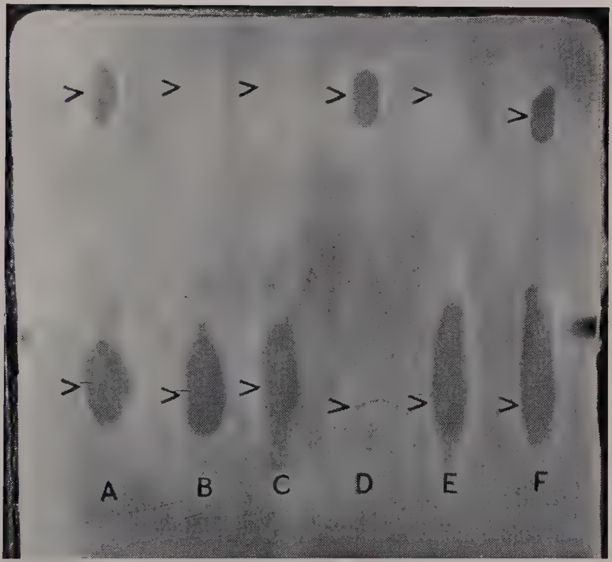

Fig. 4

Kathleen Crawford *et al.*—*Cephalosporium* antibiotics. Plate 1

Fig. 4. Zones from paper strips developed with amyl acetate, on agar seeded with *B. subtilis* 288. Upper arrow indicates point of application; lower arrow indicates solvent front. *A*, culture filtrate showing both zones; *B*, culture filtrate after treatment with 'penicillinase' from *B. subtilis* G.B.S.2; *C*, culture filtrate after storage for 3 hr. at pH 2; *D*, aqueous residue after extraction of culture filtrate with amyl acetate; *E*, amyl acetate extract from *D*; *F*, an amyl acetate extract showing two P zones.

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An Agar Diffusion Method for the Assay of Nisin

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SUMMARY: The diffusion rate of nisin through agar at pH 7·0 is increased by the addition of a non-ionic surface active agent to the medium. A linear relationship exists between log units nisin/ml. and inhibition zone depth over the range 100–5000 units/ml. By using seeded agar in small glass tubes and delaying growth of the test organism for 24 hr., a convenient form of assay is available for dealing with large numbers of samples.

EXPERIMENTAL

Using the Oxford cylinder technique, no zones of inhibition were obtained when plates were incubated immediately after setting up, but when growth of the test organism was delayed by refrigeration for 24–48 hr. to allow time for the nisin to diffuse, some inhibition was apparent after subsequent incubation. Low concentrations of Tween 80 (Atlas Powder Co.) in the medium (Stansly & Schlosser, 1947) considerably increased the diffusion rate of nisin in agar. Some compounds of the Crill series (non-ionic surface active agents manufactured by Croda Ltd., Goole, Yorks.) had an even greater effect (Fig. 1). Thus it became possible, by incorporating 0·1 % Crill 20 in the medium, to carry out agar diffusion assays provided that the plates were held in the refrigerator for 24 hr. after setting up and before incubation. An adaptation of the method of Mitchison & Spicer (1949), using narrow glass tubes containing nutrient agar seeded with the test organism, was found to be much more convenient than the use of plates.

Procedure

The routine finally adopted was as follows.

Test organism. Streptococcus agalactiae was used.

Medium. Peptone (Oxoid), 10 g.; Lab Lemco, 5 g.; sodium phosphate ($Na_2HPO_4.12H_2O$), 20 g.; sodium chloride, 20 g.; dissolved in 500 ml. tap water, boiled at pH 8·4 and filtered. To this was added an equal volume of 1 % New Zealand agar in tap water, together with glucose, 5 g., and Crill 20, 0·5 g. The reaction of the complete medium was adjusted, if necessary, to pH 8·0. This fell to pH 7·4 during sterilization.

Diluent. 0·02 N-hydrochloric acid. This gave a standard response gradient equivalent to the best of those obtained with buffer solutions at various pH levels (Fig. 2).

Tubes. 100 × 7 mm. glass tubes (Franco British Electric Co.) fulfilled the requirements of having sufficient diameter to facilitate filling, cleaning and pipetting, of being robust enough to stand considerable handling, and of being economical in agar consumption. It has been reported (Hirsch, 1950) that nisin adheres to glass surfaces from which it is extremely difficult to remove. Attempts to produce this condition experimentally failed, and no nisin could be demonstrated in tubes which had been in use for the equivalent of fifty assays, during which time the tubes were washed only in plain water. Ordinary laboratory detergents were used for the routine cleaning of all glassware, but if not rinsed very thoroughly, residues of detergent caused distortion of inhibition zones.

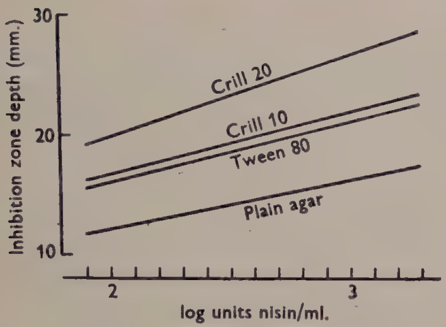

Fig. 1

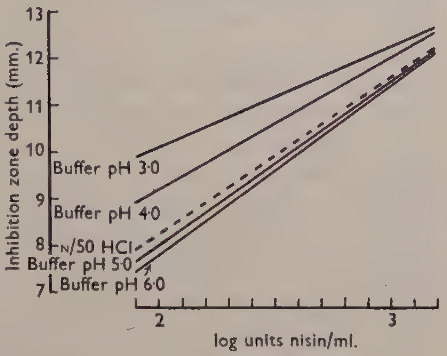

Fig. 2

Fig. 1. Effect of Tween 80 and Crill 20 on diffusion of nisin through agar. (Crill 10 is chemically identical with Tween 80.) Agar plates and porcelain cups were used in this experiment.

Fig. 2. Mean slopes of results from dilutions of nisin prepared in phosphate-citric acid buffers of different pH values and in 0·02 N-hydrochloric acid.

Racks. Strips of ⅜ in. Perspex approximately 12 × 2½ in., drilled with four rows of eighteen holes and sealed on the under side with strips of ⅛ in. Perspex.

Standard. Powder 118 K was assigned a value of 10 million units/g. based on the original definition of the nisin unit (Mattick & Hirsch, 1947); it was stored in a desiccator at 5° as a master standard. Other purified powders were used as subsidiary standards, fresh solutions containing 10,000 units/ml. were prepared each week in 0·02 N-HCl and stored at 5°. The responses of a series of dilutions of nisin from 0 to 10,000 units/ml. were plotted. A marked bend in the slope was apparent (Fig. 3) so that it was decided to use two standard ranges, 0–100, and 125–2000 units/ml., according to the estimated potency of the samples to be tested.

Method

The tubes (unplugged) are sterilized and two-fifths filled with molten agar previously cooled to 43° and seeded with 1 % (v/v) of a 6 hr. culture of *Strep. agalactiae* grown in glucose Lemco peptone broth. The tubes are stood upright until the agar has set, after which they may be stored in the refrigerator until required. When dealing with large numbers it is convenient to fill tubes on one day for use on the next.

Five serial dilutions of the standard are prepared ranging from 2000 to 125 units/ml.; for low potency samples the range is from 100–2 units/ml.; 0·1 ml. or more of each of the standard dilutions is added to three sets of four tubes, so that the final arrangement of the standard rack is three blocks of tubes each containing four replicates of the five dilutions.

Unknown solids are dissolved in 0·02 N-HCl, while brews and extraction liquors are adjusted to *c.* pH 2·0 with HCl, using thymol blue and a spotting tile. Up to five dilutions in powers of 2 are then prepared according to the degree of accuracy required, and so that the estimated potency of the dilutions falls within the standard range; 0·1 ml. or more of each dilution is then added to four seeded agar tubes.

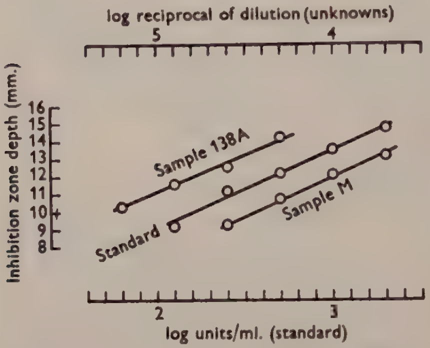

Fig. 3

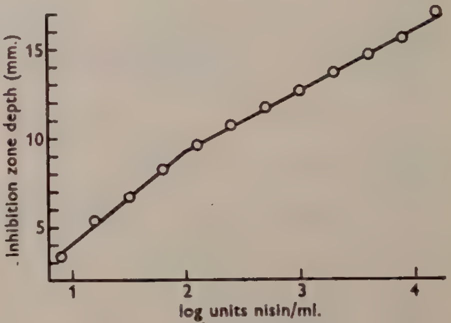

Fig. 4

Fig. 3. Mean slope of 10–10,000 units nisin/ml.

Fig. 4. Typical assay results plotted for calculation.

Pasteur pipettes are used for dispensing solutions into the seeded agar tubes, and by rinsing the pipettes twice in 0·02 N-HCl and once in the next sample to be handled, pipette consumption is decreased to a minimum. Using this technique, no carry over of nisin could be demonstrated experimentally, and it is thus possible to deal with large numbers of samples in a short space of time. Fresh standards are put up every hour when the time of setting up assays extends beyond this period. All the tubes are left in the refrigerator for 24 hr. to allow nisin to diffuse, and are then incubated at 37° overnight.

Zone readings are usually made by direct vision with the aid of a millimetre scale marked on Perspex and with top illumination against a dark background. Other methods of reading using various means of magnification have been used, but for most purposes the advantage of speed outweighs that of slightly increased precision. Differences of 0·25 mm. can be detected without undue strain.

Averages of the readings of zone depth from each set of four tubes in the standard rack are plotted against log units nisin/ml., a straight line being drawn as the mean of these points. Readings from tubes containing unknown solutions are plotted in a similar manner on the same graph, but against the logarithm of the reciprocal of the dilution. The sum of log units/ml. from the standard slope, and log dilution from the slope of the unknown, at any suitable level, is the antilog of the potency of the sample (Fig. 4). Parallelism of the slopes is an indication of the validity of the assay; to eliminate error due to lack of parallelism, readings are taken at a level which intersects the middle of the slope of the unknown.

DISCUSSION

This form of assay has been used successfully for a considerable time, and except for the additional 24 hr. delay in obtaining results it has many advantages. Although the assay tubes are sterilized before being filled with seeded agar, they are not at any time plugged nor are any aseptic precautions taken in carrying out the assay. Pipettes, dilution tubes, etc., are not sterilized, and no trouble with contamination has been encountered over a long period. The volume of solution placed on the agar column in the assay tubes is immaterial so long as this is more than 0·1 ml.; with smaller volumes some absorption takes place. The volume of agar in the tubes is unimportant so long as it is sufficient for the greatest depth of inhibition zone expected, and as the ratio between surface area and available agar volume is always constant, slight differences in tube diameters do not introduce any error. Seeded agar tubes may be stored in the refrigerator for at least a week without deterioration and are thus available at any time. This is in contrast to the standard cup-plate method where agar depth, cup diameter, etc., can affect the accuracy of the assay. Handling of tubes of this size is more convenient and they are far less bulky than Petri dishes or other types of agar plates in common use. The assay may be interpreted by the usual statistical procedures; thirty repetitions on one sample which was included daily along with routine samples gave a standard error of 5 % ($P=0{\cdot}95$).

I wish to thank Dr R. Falconer tor encouragement and interest in this work, and to express appreciation to colleagues and staff for helpful criticisms and technical assistance. Publication is by permission of the Directors of Fisons Ltd.

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Hills, G. H. the late & Spurr Errol D. (1952). *J. gen. Microbiol.* 6, 64–73.

The Effect of Temperature on the Nutritional Requirements of *Pasteurella pestis*

By The Late G. M. HILLS and ERROL D. SPURR

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SUMMARY: The nutrition of three virulent and three avirulent strains of *Pasteurella pestis* has been studied at temperatures between 23 and 37° on a basal medium containing glucose, ammonium and other inorganic salts. The organism has considerable synthetic powers and the distinction between essential nutrients and non-specific stimulants of growth is not always definite. At 32° and below, the optimal medium contained phenylalanine, valine, isoleucine, cysteine, methionine and haemin. Five out of the six strains utilized leucine in place of valine, but the maximum count was then delayed. At 36° the optimal medium contained in addition, alanine, leucine, serine, threonine, biotin and pantothenate. Omission of alanine or leucine delayed growth without reducing the maximum population. When biotin and pantothenate were omitted the organism required a mixture of twenty amino-acids.

The nutrition of *Pasteurella pestis* has been studied with conflicting results by Rao (1939, 1940*b*), Berkman (1942), Doudoroff (1943) and Herbert (1949). Rao (1939), using large washed inocula (*c.* 10^7 cells/ml.) at 27°, claimed that the only amino-acids for which there was a specific requirement were phenylalanine, proline and cystine, but that glycine was stimulatory and at least two of the four comprising alanine, leucine, lysine and arginine were also required. Metabolic experiments (Rao, 1940*a*) showed that arginine and lysine were not appreciably oxidized and these were then omitted from media used for investigating the stimulatory effects of some of the then-known growth factors (Rao, 1940*b*), but other amino-acids oxidizable by the organism were also added, viz. valine, isoleucine, methionine, serine, tyrosine and glutamate. Doudoroff (1943), working at 29°, claimed that only cystine and phenylalanine were essential on first transfer from blood agar, although proline stimulated two pathogenic strains. A non-pathogenic strain was able to utilize thiosulphate, sulphite, thiolacetic acid or homocystine, but not methionine, in place of cystine. Large inocula were necessary, however. Berkman (1942), using an inoculum of $1\text{–}2 \times 10^4$ cells/ml. and a temperature of 37°, found that four out of five strains grew in 1–2 days on a mixture of 18 α-amino-acids or a gelatin hydrolysate medium, but the fifth strain failed to grow under these conditions. Using a similar medium containing 20 α-amino-acids, Herbert (1949), also working at 37°, found growth to be irregular even with as large an inoculum as 10^6 organisms/ml., but the addition of haemin led to consistent growth from as few as 10 cells/ml.

Rao (1939) and Doudoroff (1943), working below 30° in the neighbourhood of the recognized optimum temperature, agree in claiming simpler nutritional requirements than those observed at 37° by Berkman (1942) and Herbert (1949). The observations of the two former authors are of little value, however, owing to the use of large inocula. It was considered desirable therefore to

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re-investigate the nutritional requirements of *Past. pestis* at 27–29° using small inocula. Although, in general, it may be of little value to study the nutritional requirements of an organism at a temperature removed from the optimum, the problem of the nutrition of *Past. pestis* in the neighbourhood of 37° was also studied further on account of the virulence of the organism for some warm-blooded animals and because growth at this temperature is of importance for the production of the prophylactic envelope antigen of Schütze (1932, 1939).

METHODS

In general, the methods were those of Herbert (1949) modified as follows.

Glassware. After the usual chemical cleaning in chromic-sulphuric acid, hot where possible, glassware was rinsed freely with tap water and distilled water, soaked in distilled water for several hours to remove adsorbed chromium and finally rinsed several times in glass distilled water.

Amino-acid media. Constituents were sterilized separately (hydroxy- and sulphur-containing compounds by Seitz-filtration) and mixed aseptically just before use. Glucose (Seitz-filtered) was present in the media for all experiments reported, but to avoid excessive development of acidity during growth the concentration was decreased to 5 mM. For the same reason, the ammonium sulphate concentration was decreased to 2 mM in most experiments where it was used. In some experiments it was replaced by ammonium lactate, 4 mM., until this was found to be disadvantageous rather than beneficial. The concentrations of amino-acids were (mM.): glycine, 1·4–2·0; DL-alanine, 1·0–2·0; DL-valine, 1·0; DL-leucine, 1·0; DL-isoleucine, 1·0; DL-norleucine, 1·0–2·0; DL-serine, 1·0; DL-threonine, 1·0; DL-methionine, 0·4–1·0; DL-homocystine (S), 0·4; DL-cysteine, 0·4–0·5; DL-cystine (S), 0·4; DL-aspartic acid, 1·0–2·0; DL-glutamic acid, 8–10; DL-lysine, 1·4; L-arginine, 0·7; L-histidine, 0·7–1·0; DL-proline, 1·4–2·0; L-hydroxyproline, 0·7; DL-phenylalanine, 4·0; DL-tyrosine, 0·7; and DL-tryptophan, 0·3 [(S) signifies a synthetic preparation]. The concentrations of other growth factors were: biotin, 0·005 μM.; folic acid, calcium pantothenate, riboflavin and aneurin 1·0 μM.; pyridoxin, 2·0 μM.; *p*-aminobenzoate and nicotinamide, 5 μM.; choline, haemin, inositol and pimelic acid, 10 μM.; adenine, guanine, xanthine, cytosine, thymine, uracil and glutamine, 50 μM.; glutathione, 200 μM. The concentrations of the cations were: Fe^{++} (as $FeSO_4.(NH_2)_2 SO_4.6H_2O$) 0·5 μM.; Mg^{++} (as $MgSO_4$), 200 μM.; Ca^{++} (as $CaCl_2$), usually 10 μM. but sometimes increased to 100 μM., when 1 mM. sodium citrate was added to hold the metal in solution; Cu^{++} (as $CuSO_4.5H_2O$), Mn^{++} (as $MnSO_4.4H_2O$) and Zn^{++} (as $ZnSO_4.7H_2O$) 2 μM. each when used, but they were usually omitted in experiments with a high Ca^{++} concentration. In general, we found it best to use all six metals. The media were buffered with 30 mM. phosphate, pH 7·6, prepared from thrice-recrystallized KH_2PO_4.

Conditions of cultivation. All cultures were incubated aerobically, 5 ml./50 ml. conical flask, in an atmosphere of 5 % CO_2 + 95 % air. They were left stagnant, since shaking, before the appearance of visible growth, was inhibitory.

Measurement of growth. Viable counts were used since turbidimetric methods were unreliable. Counts on cultures showing visible growth were carried out either at 24 hr. intervals or, for some experiments at 36° where the rise and fall in count might occur within this period, at 12 hr. intervals. Cultures showing no visible growth were usually tested weekly and incubated till no colonies were produced from 0·25 ml. of a 1/10 dilution. The agar used for counts contained tryptic digest of beef, diluted to contain 0·5 % total N, together with 1·5 % Oxoid (Oxo Ltd.) agar, 5 % peptic digest of sheep's blood and 1/500,000 crystal violet (Herbert, 1947). Plates were dried 1 hr. at 60° and inoculated with 0·25 ml. of suitable dilutions of culture, spread on the surface by means of a bent glass rod (cf. Snyder, 1947). Loss on the spreader was of the order of 1 %. The plates were incubated 40 hr. at 28°.

Using the whole surface of a 9·5 cm. diameter plate it was thus possible to count up to 400–500 colonies/plate without observing statistical evidence for overcrowding. Usually 400–750 colonies were counted on two to four plates inoculated with serial twofold dilutions, the estimated accuracy being of the order of ±13 % ($P = 0{\cdot}95$). Greater accuracy was not essential, since the counts of duplicate cultures rarely showed such small differences.

Strains. In addition to the virulent strains 327, 336 and 337 and the avirulent strains Soemedang S and Tjiwidej S used by Herbert (1949), Java S, a third avirulent strain of Otten (1936) was also used.

Standardization of inocula. Tryptic beef + peptic blood agar stabs were made direct from gelatin ascorbic acid dried stock cultures (Stamp, 1947). The stabs were preserved at 0–2° and subcultured as required on to tryptic beef agar slopes, to decrease the haemin content of the organisms. After incubation for 16–18 hr. at 37°, some of the growth was raked from the surface and emulsified in *c.* 5 ml. of 25 mM. phosphate buffer. After centrifuging and resuspending the organisms in phosphate buffer, the density of the suspension was determined nephelometrically, and dilutions made so as to give an inoculum *c.* 5×10^3 organisms/ml. culture. The inoculum size was always checked by viable count.

RESULTS

Preliminary experiments with strain Tjiwidej S showed that even with an inoculum as large as 2×10^5 viable cells/ml., the minimal 8 amino-acids of Rao (1939) were inadequate for growth at 37°. Moderate growth was observed, however, in 4–8 days at 31° when alanine and arginine were replaced by valine and isoleucine. The medium then contained: phenylalanine, valine, leucine, isoleucine, cysteine, lysine, proline. An experiment at five temperatures between 21 and 37° showed that growth was most rapid at 28° and that glycine, proline and lysine were not essential at this temperature. The temperature of 28° was therefore chosen for further investigation, although high counts were maintained longer at lower temperatures. Subsequently, the additional nutrient requirements for growth at 36° were examined.

Nutritional requirements at 28°

Avirulent strain Tjiwidej S. When the inoculum was decreased to 10^4 cells/ml. or less, an increasingly stringent requirement for haemin was observed at 28°, as had been found by Herbert (1949) at 37° for five strains, including Tjiwidej S.

The only amino-acids in the medium were now phenylalanine, valine, leucine, isoleucine and cysteine. Phenylalanine was found to be absolutely essential, but in view of the inhibition of growth with some combinations of valine, leucine and isoleucine, first shown by Gladstone (1939) with *Bacillus anthracis* and found by Doudoroff (1943) to occur with *Past. pestis*, the requirement for cysteine was tested in conjunction with all combinations of these unsubstituted amino-acids. With the complete mixture growth was only moderate and not always reliable. The only other combination which supported growth was that containing valine and isoleucine, but this only did so in three out of twelve experiments.

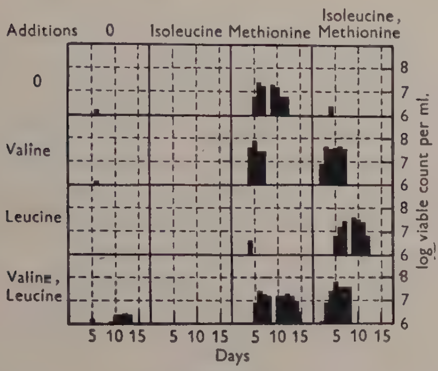

Fig. 1

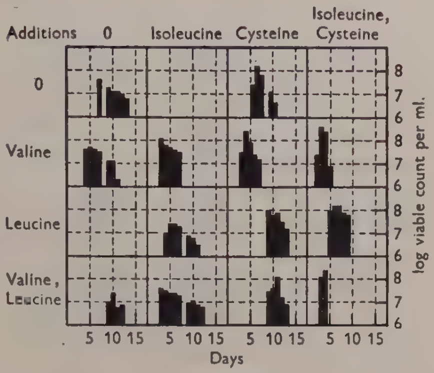

Fig. 2

Fig. 1. Inter-relationships between requirements for valine, leucine, isoleucine and methionine at 28°. Strain: Tjiwidej S. Basal medium: phenylalanine, haemin. No counts were made at 8 days.

Fig. 2. Inter-relationships between requirements for valine, leucine, isoleucine and cysteine in the presence of methionine at 28°. Strain: Tjiwidej S. Basal medium: phenylalanine, methionine and haemin. No counts were made at 8 days.

Investigation of other sources of sulphur failed to confirm the findings of Doudoroff that methionine was not only ineffective, but even inhibited the utilization of cysteine. We found, on the contrary, that after some delay methionine and phenylalanine alone supported moderate growth, which was only slightly improved by the addition of valine and was inhibited by the separate additions of leucine and isoleucine (Fig. 1). The addition of valine plus isoleucine, however, gave markedly earlier growth.

When the methionine was supplemented as sulphur source with cysteine, the results were even better than with methionine alone. Using these two sources of sulphur together, leucine and isoleucine again proved to be inhibitory, whilst the addition of valine plus isoleucine gave a good peak count of over 10^8 cells/ml. on the second or third day (Fig. 2). In other experiments alanine was found to be inhibitory, especially with suboptimal combinations of valine, leucine or isoleucine.

Homocystine was suitable as a substitute for methionine except in the presence of cysteine (Fig. 3). The delay in growth caused by this substitution was diminished in the presence of cystine or glutathione. Unless the source of sulphur is adequate for rapid growth, however, results tend to be variable; thus in the presence of phenylalanine, valine, isoleucine and cysteine, growth sometimes occurs, and sometimes fails (Fig. 3). For this reason we consider methionine and cysteine together as the most suitable source of sulphur for *Past. pestis.*

During these experiments the requirement for haemin and phenylalanine was tested with a variety of combinations of the other amino-acids. Growth was never observed in the absence of phenylalanine but occurred occasionally in the absence of haemin. We also examined the effects of thiosulphate, sulphide and thiolacetate in various basal media. These compounds have been stated to act as sources of sulphur (Doudoroff, 1943), and sulphite and thiolacetate eliminate the need for haemin at 37° (Herbert, 1949). At 28° we have been unable to determine conditions under which either of these functions is performed consistently. When growth occurs it is invariably delayed and may be scanty. It sometimes occurs after the cell population has fallen to a few cells/ml. when chance variations in conditions may determine death or survival.

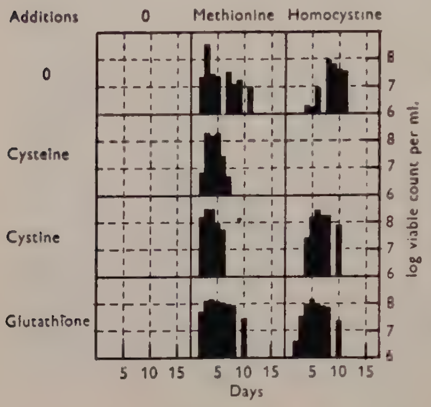

Fig. 3

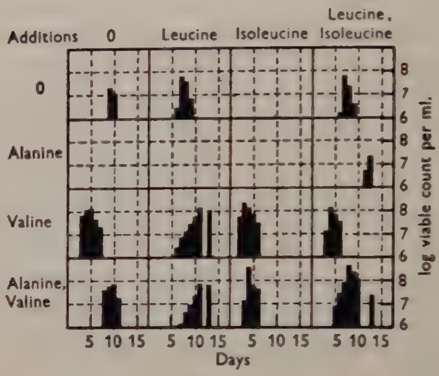

Fig. 4

Fig. 3. Sulphur requirements at 28°. Strain: Tjiwidej S. Basal medium: phenylalanine, valine, isoleucine and haemin.

Fig. 4. Inter-relationships between alanine, valine, leucine and isoleucine requirements at 28°. Strain: 337. Basal medium: phenylalanine, cysteine, methionine and haemin. No counts were made at 12 days.

Virulent strain 337. This grew with the same amino-acids as had been found best for Tjiwidej S, namely phenylalanine, valine, isoleucine, cysteine and methionine. Determination of the inter-relationships between the unsubstituted amino-acids showed that valine and isoleucine again formed the best combination (Fig. 4). Alanine was tested in this experiment and tended to inhibit rather than promote growth, especially in the absence of valine or isoleucine. The most satisfactory source of sulphur was again a combination of cysteine and methionine. As with strain Tjiwidej S, cystine was utilized as effectively as cysteine, but substitution of glutathione for cysteine or of homocystine for methionine, delayed growth for 1–4 days. The need for phenylalanine and haemin was also established.

Other strains. The four other available strains grew to a maximum population over 2×10^8 cells/ml. in 2–4 days on the medium containing phenylalanine, isoleucine, valine, cysteine and methionine. The time taken to reach the maximum population was 3 days longer with leucine instead of valine and in one experiment Java S failed to grow in this medium.

Nutritional requirements at 36°

Avirulent strain Tjiwidej S. In the course of many experiments at 37° on a medium containing 20 amino-acids similar to that of Herbert (1949), growth was frequently delayed for 5–8 days, sometimes failed and was always poor, rarely reaching 10^8 cells/ml. Although the failures were subsequently correlated with overheating to 37·5°, consistent growth was first observed as a result of adding mixtures of growth factors, of which those of the vitamin B group, especially biotin, nicotinamide, pantothenate and aneurin were of greatest importance. Temperatures above 37° still caused erratic results and further experiments on the simplification of the supplements necessary for growth above 28° were carried out at $36{\cdot}0 \pm 0{\cdot}5°$.

Of the amino-acids, besides the five already mentioned as being essential, only a group containing alanine, leucine, norleucine, serine and threonine appeared to be necessary. Detailed examination of this group showed that alanine and norleucine could be omitted and that growth was delayed or much diminished in the absence of threonine. Investigation of the requirements for growth factors in the presence of this simplified amino-acid mixture (Fig. 5) showed that consistent growth was observed only with mixtures containing biotin and pantothenate together, delayed growth occurring with other mixtures or with single factors. In such cases the rate of growth was not only variable from experiment to experiment but even between duplicates in the same test. Whenever growth occurred, however, the maximum population was always of the order of 10^8 cells/ml. Finally, the amino-acid requirements were retested in the presence of biotin and pantothenate as the only factors of the vitamin B group. Phenylalanine, cysteine, methionine, serine and threonine, and, of the unsubstituted amino-acids, valine and isoleucine, were then essential. Alanine and leucine were stimulatory.

Virulent strain 337. This strain was grown by Herbert (1949) in media containing 12–20 amino-acids at 37°. We confirmed his results in five out of seven experiments in which the temperature did not exceed 37°. In the remaining experiments, where the temperature rose to 37·5°, the cultures rapidly became sterile, though growth occurred in our simplified amino-acid medium containing biotin and pantothenate. At 36° the optimal combination of amino-acids was that required at 28° and in addition alanine, leucine, serine and threonine (Fig. 6). Moderate growth occurred in 4–5 days, even when these latter amino-acids were omitted; leucine was the only one which by itself was stimulatory, growth occurring then after 3 days. Serine, threonine or alanine, added separately, were inhibitory. With serine plus threonine growth was good but delayed, this delay being overcome by the stimulatory effects of alanine and leucine together when a good peak count was obtained within 2–3 days. In the same experiment no growth occurred on omission of phenylalanine, biotin or pantothenate from the optimal medium. In other experiments the need at 36° for cysteine, methionine and haemin was also confirmed. Thiosulphate, sulphite or cysteine alone did not serve as sources of sulphur either with or without haemin. The virulence of strain 337 when grown on this medium at 36° was, if anything, enhanced as compared with the virulence of the same strain grown on tryptic beef broth.

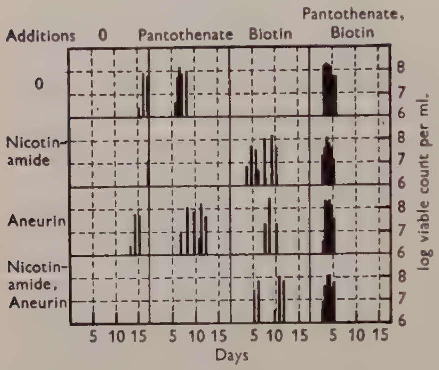

Fig. 5

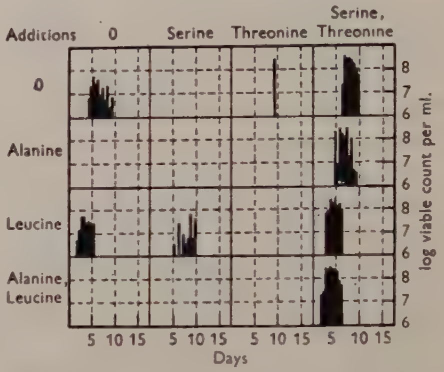

Fig. 6

Fig. 5. Requirements for biotin, nicotinamide, pantothenate and aneurin at 36°. Strain: Tjiwidej S. Basal medium: phenylalanine, valine, leucine, isoleucine, serine, threonine, cysteine, methionine and haemin. Two columns per day show variations between duplicate cultures. Counts were made at 8·5 days insted of at 8 and 9 days.

Fig. 6. Inter-relationships between requirements for alanine, leucine, serine and threonine at 36°. Strain: 337. Basal medium: phenylalanine, valine, isoleucine, cysteine, methionine, haemin, biotin and pantothenate. Two columns per day show variation between duplicate cultures.

Other strains. The other four strains available grew in our optimal medium containing phenylalanine, alanine, valine, leucine, isoleucine, cysteine, methionine, biotin, pantothenate, serine, threonine and haemin. All except Java S reached a maximum population exceeding 2×10^8 cells/ml. in 2 days. The virulent strains 327 and 336 reached this count in 7 days with haemin and the 5 amino-acids, phenylalanine, valine, isoleucine, cysteine and methionine. Valine was not replaceable by leucine under these conditions.

Nutritional requirements at other temperatures

Fig. 7 shows the effect of various media on the growth of strain 337 between 23 and 36°. The leucine + serine + threonine mixture was inhibitory or delayed growth at the lower temperatures though it was essential for early growth at 36° in conjunction with biotin and pantothenate or 12 other amino-acids. This inhibition was also observed with strain Tjiwidej S but has not been further investigated.

DISCUSSION

So far as we are aware, effects of temperature on the nutrient requirements of micro-organisms have been observed previously only with *Neurospora* mutants, twelve such temperature-sensitive mutants being recorded by Beadle & Tatum (1945). Details of a mutant requiring riboflavin at 28–32° have been given by Mitchell & Houlahan (1946). Emerson (1947) described a mutant requiring sulphonamides at 35° and a double mutant requiring sulphonamides and *p*-aminobenzoate at 35°. Such effects have not been established previously with bacteria in spite of active investigation, especially by workers with thermophilic organisms (Gaughran, 1947; Cleverdon, Pelczar & Doetsch, 1949).

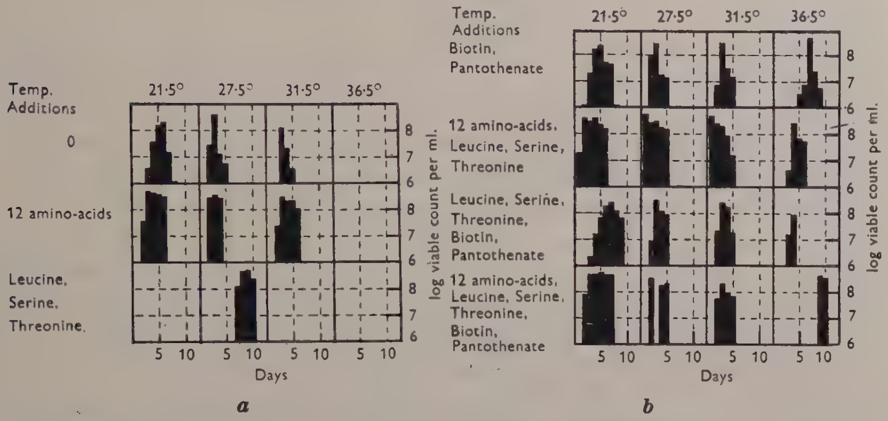

Fig. 7. Effect of temperature on growth in various chemically-defined media. Strain: 337. Basal medium: phenylalanine, valine, isoleucine, cysteine, methionine and haemin. Twelve amino-acids: glycine, alanine, norleucine, aspartate, glutamate, lysine, arginine, histidine, proline, hydroxyproline, tyrosine and tryptophan. Fifteen amino-acids: 12 amino-acids, leucine, serine, threonine.

Our results at 36° agree with those of other workers at 37° (Berkman, 1942; Herbert, 1949) in so far as we get satisfactory growth with 20 amino-acids as source of nitrogen. We find, however, that with such a medium growth does not occur when the temperature rises to 37·5°. More consistent growth is observed when 12 of these amino-acids are replaced by biotin and pantothenate. Regarding the remaining 8 amino-acids which we consider essential for satisfactory growth at 36°, Herbert (1949) found omission of serine or methionine to delay growth slightly, omission of threonine to delay growth markedly and omission of valine, leucine, isoleucine, cystine or phenylalanine to prevent growth. We have generally used cysteine instead of cystine, since synthetic DL-cysteine was available and it showed little difference in behaviour from cystine of natural origin (L), except in the presence of homocystine. At 28° we found the most satisfactory medium to contain 4 of the amino-acids which Herbert (1949) found to be the more important, leucine, however, being replaced by methionine. This medium is more complex than that suggested by Doudoroff (1943), who considered only phenylalanine and a source of sulphur to be essential in a medium containing ammonium salts. The probable reason for this discrepancy is that Doudoroff used large inocula.

The organism has considerable synthetic ability. With small inocula results tend to be variable and it has been difficult to distinguish between essential and stimulatory amino-acids. Accordingly, we have regarded with suspicion late growth which failed to reach a maximum population of 10^8 cells/ml. We have only regarded a medium as adequate when the growth was practically as heavy (*c.* 2 to 6×10^8 viable organisms/ml.) as that produced in 1 % tryptic casein digest.

In this study we have established the qualitative nutritional requirements of six strains of *Past. pestis*, but in all probability we have not employed our growth factors in their optimal concentrations. The results obtained would permit a determination of these optimal concentrations; it is very probable that a balanced medium would support more rapid and abundant growth, and that the medium might even be further simplified.

Further work will be necessary before it is possible to explain why the organism is nutritionally more exacting at 36° than at lower temperatures. In view of the connexion between biotin and the deamination of aspartate, serine and threonine (Lichstein & Christman, 1948), it is tempting to suppose that biotin may facilitate the reverse process and catalyse the synthesis of these amino-acids. This may be true of aspartate, but is unlikely with serine and threonine, since these should be supplied for early growth at 36° in the presence either of biotin and pantothenate or of a wider array of amino-acids. It is possible that the supply of biotin or the nutrients for its more rapid synthesis may even increase the need for serine and threonine by facilitating their destruction. The function of pantothenate as a co-factor in the oxidation of fragments containing 2 C atoms, through the citric acid cycle, is rapidly becoming established, especially through the work of Lipmann and co-workers (e.g. Novelli & Lipmann, 1950). It is possible that the primary function of biotin may also be concerned with energy production in this cycle in view of its part in oxaloacetate synthesis (Wessman & Werkman, 1950). Work on the applicability of these ideas to the nutrition of *Past. pestis* may show whether the vitamins are specifically concerned in amino-acid synthesis or whether they are involved indirectly through energy yielding processes. Conversely, if additional amino-acids are supplied instead of biotin and pantothenate, it would be interesting to know whether the requirements for these two vitamins, presumably satisfied by synthesis, are comparable with those needed when the supply is exogenous.

The effects of temperature on nutritional requirements appear to us to be due to the enzymic processes which lead to the synthesis of essential metabolites being better co-ordinated at the optimum temperature. At higher temperatures, katabolic processes leading to the death of the organism are accelerated relative to synthetic processes and the organism becomes dependent on a wider range of preformed nutrients. An alternative explanation, the selection of mutants of differing requirements seems to us unlikely since those cultures which grow at 36° begin to do so with little lag even though growth may be slow for several days, while those cultures which fail to grow rapidly become sterile. The selection of mutants is more likely in those cultures on suboptimal media at lower temperatures, in which delayed growth appears some time after the count has fallen to a few cells per ml.

We wish to thank Dr D. W. Henderson and Dr D. Herbert for helpful discussion of this problem. Publication is by permission of the Chief Scientist, Ministry of Supply.

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The Action of Plant Extracts on a Bacteriophage of *Pseudomonas pyocyanea* and on Influenza A Virus

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SUMMARY: Extracts of 288 plants, mostly British, were examined for suppressive action on the development of a bacteriophage of *Pseudomonas pyocyanea*. Many possessed this property and eight of them suppressed the growth of phage at concentrations less than one-tenth of those which affected the growth of the host, *Ps. pyocyanea*.

Extracts from 142 of the plants were tested against Influenza A virus in embryonated eggs and twelve of them suppressed virus multiplication. All extracts active against Influenza A virus were also active against the bacteriophage. Four extracts tested against Influenza A in mice were inactive. Eight extracts were investigated further; these were inactivated by proteins and were only active when in direct contact with the virus in protein-free media. Activity was closely associated with the tannin content of the extracts and could not be separated from it. Commercial tannins were also highly active in protein-free media.

During investigations into the chemotherapy of virus diseases a phage of *Ps. pyocyanea* had been used as a convenient test virus by one of us (Dickinson, 1948) for screening a large number of substances including fifty plant extracts. With certain modifications this work was extended and extracts of 288 plants, mostly British, were investigated. A complete list of the species tested, with their activities, appears in Table 7. The following eight plants were investigated in detail in an attempt to isolate the active principle and elucidate the mode of action: no. 9, *Dryopteris filix-mas* (Schott); no. 20, *Epilobium hirsutum* L.; no. 32, *Lysimachia Nummularia* L.; no. 84, *Epilobium angustifolium* L.; no. 98, *Sedum spurium* M. Bieb.; no. 240, *Lysimachia vulgaris* L.; no. 250, *Saxifraga ligulata* Wall.; no. 287, *Myrica Gale* L. (The numbers correspond with those in Table 7.) So far as possible, with British species, names are in accordance with the nineteenth edition (1948) of Hayward's *Botanist's Pocket Book*.

At some stages of our work the identification of the samples was unable to keep up with the rate of supply, and although a portion of the plants was always kept for examination it was not always possible to decide upon the specific name. Difficulty also arose when an extract from a florist's variety was under test. In both these cases the generic name followed by (?sp.) appears in Table 7. In some cases several varieties of the same species were tested.

METHODS

Preparation of extracts

Plants in as fresh a condition as possible were macerated in distilled water plus 15 % (v/v) industrial spirit, using a bench macerator with high-speed blades, to make 10 % (w/v) suspensions. The alcohol was used as a preservative, 15 % being the maximum concentration which did not interfere with the tests; bacterial growth was inhibited but moulds occasionally grew on storage. The extracts were usually tested the day after preparation. As far as possible, samples representative of the whole plants were taken, as the concentration of active material in the different parts varied. There was also some variation between samples of the same plant when collected at different times.

Testing of extracts

Against Ps. pyocyanea *phage* in vitro. The method was that of Dickinson (1948) with certain modifications. Dilutions of the extracts were made in 9 ml. lactate medium, 0·5 ml. 1/10 dilution of a 24 hr. culture of *Ps. pyocyanea* (C10) was added, followed at once by 0·5 ml. phage (Pb) suspension with a titre of about 7000 infective phage particles per ml. The tubes were incubated at 37° for 20 hr. and a loopful from each was then placed on a seeded plate prepared by pouring a thin layer of agar containing 5 % (v/v) C 10 culture on to a layer of nutrient agar and then drying. About thirty-seven 'spots' were placed on each plate. The plates were incubated for 20 hr. and then examined. When no bacteriophage suppression occurred in the dilution tubes, the 'spotted' area showed confluent lysis and such dilutions were described as inactive. Dilutions were described as active when no such lysed area formed, provided that the growth of host at that dilution was not affected. The original concentration of phage in the dilutions was insufficient to produce an area of lysis on the plate. Host growth in the dilution tubes was occasionally difficult to estimate due to the presence of the plant debris; usually the formation of a surface pellicle was used as the criterion of host growth but when no pellicle formed, bacterial counts were made by the plate method.

Against Influenza A virus in eggs. Extract (0·2 ml.) was inoculated into the allantoic sac of 11-day chick embryos; 1 hr. later, approximately 100 minimal infective doses of Influenza A virus (strain PR 8) in 0·2 ml. phosphate buffer (pH 7·4) were inoculated by the same route, followed by 100u. each penicillin and streptomycin in 0·1 ml. water. After a further 48 hr. incubation at 37°, the increase of virus in the allantoic sac was detected by the haemagglutination of fowl red blood cells, based on Salk's method (1944). One drop of allantoic fluid was added to 0·5 ml. 0·25 % washed fowl cells in saline, and the mixture allowed to stand at room temperature for 1 hr. Haemagglutination was considered an indication of virus multiplication, as the inoculum was insufficient to cause haemagglutination; neither false haemagglutination nor 'haemagglutination inhibition' was observed in control tests with extracts at the concentrations used. Extracts were also inoculated at varying intervals after infection and via the yolk sac 1 hr. before infection.

Against Influenza A virus in mice. The maximum tolerated dose of extract (containing about 10 times the amount found active in the embryonated-egg test) was given subcutaneously twice daily, beginning immediately before infection. Ten to one hundred infective doses of Influenza A virus were given intranasally under light ether anaesthesia. The survivors were killed 7 days after infection and the lungs examined. In one test a 1 % solution of an extracted solid was given as an aerosol for 1 hr. a day, starting before infection.

EXPERIMENTAL

Of the 288 extracts 146 were inactive by the phage test and were not investigated further; 142 were examined both by the phage test and the Influenza A test and of these, forty-four were inactive by both tests; ninety-nine showed activity, usually slight, against phage and twelve were active against Influenza A. Of these twelve, eight had a titre of 10 or more (titres are the reciprocals of the dilutions), when tested against phage (Table 1).

Table 1. *Effect of plant extracts on the growth of viruses*

Ref. no.	Name	*Ps. pyocyanea* phage. Inhibitory titres		Influenza A virus. Inhibitory titres when injected 1 hr. before infection
		For host	For phage	
95	*Potentilla Anserina* L.	<5	5	1
250	*Saxifraga ligulata* Wall. (*)	50	450	5
98	*Sedium spurium* M. Bieb.	10	150	1
84	*Epilobium angustifolium* L.	<5	5	1
20	*E. hirsutum* L.	<5	5	1
218	*Fuchsia* (hardy garden)	?5	10	1
32	*Lysimachia Nummularia* L.	<5	100	1
240	*L. vulgaris* L. (†)	<5	100	1
43	*Polygonum amphibium* L.	<5	5	1
144	*Rumex* (? sp.)	5	10	1
287	*Myrica Gale* L.	10	30	Toxic 1; Inactive 2
276	*Scilla nutans* (‡)	<5	5	1
9	*Dryopteris Filix-mas* (Schott)	<10	100	1

(*) All garden varieties of the Megasea section of *Saxifraga* tested, labelled *Saxifraga cordifolia*, *S. crassifolia*, etc., were active.

(†) Good activity was shown by wild and garden plants. The wild plant appeared to be rather better than the garden ones; the best of the latter we examined was supplied to us as 'var. aurea'.

(‡) A garden variety supplied under this name.

Two plants were examined to ascertain distribution of the active material throughout the plant. In the case of *Epilobium angustifolium* (84), tested against Influenza A virus in eggs, the higher titres were obtained with extracts from fruits, flowers and leaves, in that order; the stems were inactive. In the case of *Saxifraga ligulata* (250), tested against phage, the highest activity was obtained with extracts from the rhizome, but this distribution may have been due to the material having been collected in autumn.

Where one plant yielded an active extract there was a tendency for related plants to do so. The best example of this is in the Onagraceae where two species of *Epilobium* and one of *Fuchsia* showed activity in both tests; *Circaea lutetiana* and *Oenothera biennis* also showed activity in the phage test, as did *Singhara* seeds. In the Primulaceae marked activity was shown by *Lysimachia Nummularia* and *L. vulgaris* in both tests and *L. nemorum* was active against phage. All the common garden Saxifrages of the *Megasea* type which were examined were highly active.

Preparation of active solids

Plant no. 250 was macerated in distilled water containing 15 % industrial spirit to make 10 % (w/v) suspensions. For convenience this preparation was left overnight and the plant debris removed by filtration through kieselguhr. The brown filtrate was adjusted to pH 2·5 with HCl and then well agitated with 1 % (w/v) activated carbon. The carbon cake after vacuum filtration was drained and stirred with 10 times its weight of acetone containing 20 % water for 20 min., the carbon filtered off and the acetone eluate shaken with an equal volume of carbon tetrachloride. The activity concentrated in the aqueous phase from which solid material was obtained by evaporation *in vacuo* at 45°. This process yielded solid material from nos. 9, 20, 84 and 250, but not from nos. 32, 93, 240 and 287. In the case of those plants which did not yield an active solid, the active material appeared to have been absorbed on to the carbon but was not removed by the acetone elution. Solutions of the solids are referred to as 'concentrates' and preparations made in the course of the extraction are termed 'fractions'.

Investigation of active materials

Phage tests in the presence of protein. When tests were carried out in nutrient broth or in lactate medium containing 10 % serum, all extracts and concentrates were inactive. The results obtained with the solids appear in Table 2.

Table 2. *Results of extraction process on active substances*

Ref. no.	Description of solid obtained	Yield (g./100 g. fresh plant)	Phage titres	
			In lactate	In broth
9	Dry, light brown amorphous powder	0·43	30,000	<10
84	Dark brown, gummy	0·67	<10	—
240	None	—	—	—
20	Dark brown, gummy	0·54	300	<10
93	None	—	—	—
32	None	—	—	—
250	Buff, amorphous powder	0·72	50,000	<10
287	Very small yield of dark brown, gummy material	—	—	—
Malabar kino	Dark, reddish brown powder	6·70	100,000	<10
Tannic acid	Untreated B.P. material	—	10,000	<200

Effect of broth and serum on the action against Influenza A virus. The extracts or concentrates were diluted in broth or 10 % serum before inoculation into the egg. When precipitation occurred the supernatants after centrifugation were used. Substances so treated were inactive.

Tests in egg. Extracts which were active when given 1 hr. before infection were also active when given 1 hr. after infection. In one experiment with no. 84, activity was demonstrated when given 4 hr. but not 5 hr. after infection. The extracts were inactive when given via the yolk sac.

Tests in vivo. The extracts were inactive against influenza A virus in mice (Table 3).

Table 3. *Action of plant extracts against Influenza A virus in mice*

Test	Test material	Survivors/total 7th day	Remarks
1	No. 84 (extract)	1/9	Toxic dose. All lungs infected
	Controls	5/10	All lungs infected
2	No. 250 fraction	4/10	All lungs infected
	No. 240 fraction	5/10	All lungs infected
	Controls	4/10	All lungs infected
3	Malabar kino concentrate (1 % aerosol)	7/11	All lungs infected
	Controls	4/8	All lungs infected

Phage contact test. Solutions (1 %) of the active solids from extracts 240 and 250 and crude extracts of 84, 98, 240 and 287 were serially diluted in broth and lactate medium. Volumes (0·5 ml.) of dilutions were left in contact with 0·5 ml. phage preparations of known titre for 30 min. at room temperature. After this time, 0·5 ml. host culture in nutrient broth plus 4 ml. nutrient agar were added. This stopped further action on the phage in lactate medium. The tubes were 'sloped' and incubated for 24 hr., when control tubes showed a countable number of plaques.

The phage of *Ps. pyocyanea* was used in early tests but later the T 5 phage of *Escherichia coli* was used as it proved more sensitive and gave more clear cut results. Extracts of nos. 84 and 250 were tested against both *Esch. coli* and *Ps. pyocyanea* phages as well as against their hosts. For host counts, the drug dilutions were mixed with the host, allowed to stand for 1 hr., and then 'sloped' with nutrient agar. The inocula were adjusted to give discrete colonies in the control tubes.

The results given in Table 4 indicate that the extracts and concentrates inactivated the phages of *Ps. pyocyanea* and *Esch. coli* in defined simple medium when left in contact with them for 30 min. at room temperature, but they did not do so in the presence of broth.

Properties of the active solids. Solutions of the active extracted solids were precipitated by protein media, lead acetate or phenazone (1-phenyl-2,3-dimethyl-5-pyrazolone) and gave a blue coloration with ferric chloride. Similar tests to those described for the tests in eggs were carried out with extracts which had been treated with varying concentrations of phenazone to precipitate tannins. Concentrations ranged from 1 to 12 % phenazone, and after standing for 1 hr. at room temperature the tubes containing the maximum precipitate (usually about 3 % phenazone), were centrifuged at 10,000 r.p.m. for 15 min. Supernatants and precipitates were tested separately

Table 4. *Contact tests against bacteria and phage*

Test material	Medium	Titres* *Ps. pyocyanea* host	*Ps. pyocyanea* phage	*Esch. coli* host	T5 phage
No. 250 extract	Lactate	16	64	64	1024
	Broth	16	0	64	4
No. 250 solid (1 % solution)	Lactate	16	±64	4	64
	Broth	16	0	4	0
No. 84 extract	Lactate	4	±4	2	16
	Broth	16	0	2	0
Malabar kino solid (1 % solution)	Lactate	0	16	0	16
	Broth	0	0	0	0

* Titre is the reciprocal of the dilution necessary to decrease the count of the test organism to <10/tube.

in lactate medium and in broth. The results in Table 5 show that when precipitation occurred, the supernatant had little or no activity. The phenazone precipitates showed slight activity, probably due to dissociation of the complex.

Table 5. *Action of phenazone on plant extracts in contact tests*

Test material	Medium	Titre against T 5 phage: Extract	Super. after 1–3 % phenazone	Ppt. made up to volume	Remarks
No. 98 extract	Lactate	8	4	2	Only slight ppt.
	Broth	0	0	0	
No. 240 extract	Lactate	32	4	32	Heavy ppt.
	Broth	2	0	0	
No. 287 extract	Lactate	32	2	±10	Variable ppt.
	Broth	2	2	0	
No. 84 extract	Lactate	16	0	8	Heavy ppt.
	Broth	0	0	0	
Malabar kino solid (1 % solution)	Lactate	100	0	2	Heavy ppt.
	Broth	5	0	0	

Investigation of the tannins

In view of the properties, source and mode of extraction of the active solids, it was suggested that the activity was due to tannins. Several samples of commercial tannins were therefore investigated in both lactate medium and broth and against Influenza A virus (Table 6). All extracts were inactive against phage in broth. Malabar kino was extracted by the same method as the plants and yielded a very potent solid; this was also inactive in broth. The malabar kino concentrate was inactive *in vivo*. It behaved similarly to the plant extracts when tested in a contact test against *Ps. pyocyanea* and *Esch. coli*, phages before and after phenazone treatment.

Table 6. *Effect of extracts of pharmaceutical tannin-containing drugs on the growth of viruses*

Sample (tested as a 1·0 % (w/v) maceration in 15 % ethanol)	*Ps. pyocyanea* bacteriophage; (titre in lactate medium)	Influenza A (titre in eggs)
Hammamelis leaves	100	1
Caryophyllum (cloves)	50	1
Gallum (oak galls)	300	nt
Cinnamon bark	30	1
Oak bark	80	1
Maté Tea leaves	50	Inactive 10, toxic 1
Cinchona bark	50	1
Malabar kino	300	Active 10, toxic 1
Eucalyptus kino	300	nt
Catechu	100	1
Krameria root	300	Active 10, toxic 1
Areca nut	500	10
Catechu nigrum	100	Active 10, toxic 1
Bengal kino	1,000	1

nt = not tested.

DISCUSSION

The complete lack of action of the plant extracts in the presence of serum or broth, the effect of phenazone treatment and the properties of the active solids suggested that all the plant extracts investigated owed their activity to their tannin content. This view was supported by the similar behaviour of commercial tannins. Although extracts of different plants did not always behave alike, e.g. in their adsorption to and elution from carbon, this phenomenon is not unexpected, 'tannins' being a descriptive term for different chemical substances. The possibility that the active material was not itself a tannin, but was carried down by the precipitates was not excluded but, in view of the behaviour of commercial tannins, this seems unlikely.

Since the plant extracts were active against phage only in protein-free medium it seemed unlikely that they would be of chemotherapeutic value; the small-scale mouse test against Influenza A virus supported this view. The action of the extracts on Influenza A virus in eggs is of interest and, early in the work, resulted in false assumptions being made as to the mode of action. The extracts were active when given 1 hr. before and 1 hr. after infection, and in one experiment extract no. 84 was active 4 hr. but not 5 hr. after infection. It was at first thought that this indicated an interference with the metabolism of virus within the host cells, but it is now evident that a viricidal action in the allantoic fluid, which contains very little protein, cannot be excluded. After 1 hr. and certainly after 4 hr. the virus would have been adsorbed on to the cells but the extracts could still exert their viricidal action when the virus was liberated into the allantoic fluid. Broth and serum-treated extracts were inactive against Influenza A when given via the allantoic sac, and the extracts showed no activity when given via the yolk sac. Similar results have been reported by Green (1948), who found that tannic acid prevented the multiplication of Influenza A virus in the allantoic sac when given up to 6 hr. after infection; the virus was inactivated *in vitro*. Green (1949) also found that tea extracts were active via allantoic sac 2 hr. after infection but were inactive via the yolk sac. One of the incidental aims of these investigations was to establish a correlation, if any, between the phage test and the Influenza A test. Since the active principles of all the plants were probably acting by a similar mechanism, due to their tannin properties, the results do not clarify the position. However, no extract was found to be active against Influenza A without also showing activity against phage. The reverse was not true, 87 extracts showing slight activity against phage and none against Influenza A. It should be noted that in the phage test solutions are diluted 1/5, whereas in the egg test they are diluted about 1/50 by the allantoic fluid. In the absence of certain correlation, phage can only be used as one convenient test virus for 'screening' purposes. The results may or may not be applicable to other viruses.

Table 7. *List of plants (with laboratory reference numbers in italic) tested against phage. Titre, in brackets, is the reciprocal of the dilution at which the extract was active. Extracts when tested against Influenza virus A in eggs are shown when active as V+, or when inactive as V–*

Alismaceae. *5, Alisma Plantago-aquaticum* L. (5) V – ; *255, Sagittaria sagittifolia* L. (0).

Apocynaceae. *225, Vinca major* L. (5).

Araceae. *242, Acorus calamus* L. (0).

Araliaceae. *171, Hedera helix* L. (0).

Auriculariaceae. *284, Auricularia mesenterica* Bull (0).

Berberidaceae. *220, Berberis stenophylla* (5) V – ; *217, B. subcauliata* (15) V – ; *12, B. vulgaris* L. (0) V – ; *228, Mahonia aquifolium* Pursh. (0).

Boraginaceae. *289, Myosotis* sp. (5); *96, Pulmonaria officinalis* L. (0) V –.

Campanulaceae. *111, Campanula portenschlagiana* (5); *175, C. rotundifolia* L. (0).

Cannabinaceae. *259, Humulus lupulus* L. (0).

Caprifoliaceae. *210, Lonicera nitida* Wilson (0); *252, Symphoricarpus racemosus* (0); *169, Viburnum opulus* L. (0); *215, V. tinus* (0) V – ; *219, Weigelia rosea* Lindley (0).

Caryophyllaceae. *192, Dianthus barbatus* (5) V – ; *189, D. Caryophyllus* L. (5) V – ; *193, D. plumarius* L. (5) V – ; *267, Dianthus* sp. (single, garden variety) (5); *141, Lychnis alba* Mill. (0); *131, Sagina procumbens* L. (5) V – ; *55, Silene maritima* With. (0) V – ; *57, Spergularia marina* (0) V –.

Chenopodiaceae. *110, Atriplex patula* L. (5) V – ; *11, A. portulacoides* L. (0) V – ; *279, Beta* (sugar beet) (0); *280, Chenopodium* fruit (pharmaceutical) (5); *81, Chenopodium* sp. (5) V – ; *48, Salicornia herbacea* (0) V – ; *65, Suaeda fruticosa* Forsh. (0); *64, S. maritima* Dum. (5) V –.

Commelinaceae. *104, Tradescantia virginica* (0) V –.

Compositae. *2, Achillea Millefolium* L. (5) V – ; *1, A. Ptarmica* L. (0) V – ; *160, Anthemis Cotula* L. (0); *73, Arctium Lappa* L. (5) V – ; *74, Artemisia Absinthium* L. (5) V – ; *75, A. vulgaris* L. (0) V – ; *10, Aster maritima* (0) V – ; *294, Bellis perennis* L. (5); *203, Bidens cernua* L. (5); *138, Buphthalmium* sp. (garden variety) (5); *180, Callistephus sinensis* (5); *191, Centaurea Cyanus* L. (5); *15, C. nigra* L. (5) V – ; *140, Chrysanthemum Parthenium* Bernh. (0); *79, Cirsium arvense* Scop. (5); *80, C. eriophorum* Scop. (5) V – ; *139, C. lanceolatum* Scop. (5); *162, C. palustre* Scop. (5); *115, Crepis virens* L. (5) V – ; *184, Cosmos bipinnatus* (5); *159, Erigeron canadensis* L. (5); *233, Eupatorium cannabinum* L. (5); *178 Galinsoga parviflora* Cav. (5); *188, Hieracium Pilosella* L. (0) V – ; *28, Hypochaeris radicata* L. (0) V – ; *226, Lactuca muralis* Fres. (5); *123, Lapsana communis* L. (0); *149, Leontodon autumnalis* L. (5); *33, Matricaria inodora* L. (0) V – ; *34, M. suaveolens* Buch. (0) V – ; *148, Leontodon hispidus* L. (0); *264, Petasites fragrans* Presl. (0); *170, Picris Echioides* L. (5); *46, Pulicaria dysenterica* Gray (5) V – ; *52,* Table 7 (*cont.*)

Senecio aquaticus Hill (?); *53, S. Jacobaea* L. (5) V – ; *97, S. squalidus* L. (0) V – ; *132, S. vulgaris* L. (5) V – ; *167, Sonchus asper* Hill (5); *100, S. arvensis* L. (5) V – ; *153, S. oleraceus* L. (0); *179, Tagetes signata* (5); *67, Tanacetum vulgare* L. (0) V – ; *147, Taraxacum officinale* W. (5); *103, Tragopogon pratensis* L. (5) V – ; *195, Tussilago Farfara* L. (0).

CONVOLVULACEAE. *223, Calystegia sepium* Br. (5); *145, Convolvulus arvensis* L. (0).

CORNACEAE. *190, Cornus sanguinea* L. (10) V – .

CRASSULACEAE. *271, Sedum sarmentosum* (10) V – ; *266, S. spectabile* (0); *98, S. spurium* M. Bieb. (150) V + .

CRUCIFERAE. *290, Arabis albida* (5); *112, Capsella bursa-pastoris* Med. (5) V – ; *260, Cochlearia Armoracia* L. (0); *293, Iberis sempervirens* (?); *172, Lepidium ruderale* L. (0); *229, Lunaria biennis* (0); *185, Matthiola* sp. (garden variety) (5); *254 Raphanus Raphanistrum* L. (0); *152 Sisymbrium altissimum* L. (5); *133, S. officinale* Scop. (0).

CUCURBITACEAE. *137, Bryonia dioica* Jacq. (0).

CUPRESSACEAE. *216, Chamaecyparis nootkatensis* Lambert (5) V – .

DIOSCOREACEAE. *66, Tamus communis* L. (5) V – .

DIPSACACEAE. *83, Dipsacus sylvestris* Huds. (5); *187, Scabiosa arvensis* L. (0) V – ; *50, S. Succisa* L. (0) V – .

EQUISETUM. *86, Equisetum* sp. (5) V – .

ERICACEAE. *238, Calluna vulgaris* Hull. (10).

EUPHORBIACEAE. *224, Buxus sempervirens* L. (5); *119, Euphorbia Helioscopia* L. (0); *120, E. Peplus* L. (0); *239, Euphorbia* sp. (5).

FUMARIACEAE. *117, Dielytra eximia* (0).

GERANIACEAE. *23, Geranium pratense* L. (0) V – ; *244, Impatiens glandulifera* Royle (0).

GRAMINACEAE. *6, Alopeeurus pratensis* L. (0); *107, Agropyron repens* Beauv. (5) V – ; *158, Agrostis* sp. (5) V – ; *109, Arrhenatherum avenaceum* Beauv. (5) V – ; *13, Brachypodium pinnatum* Beauv. (?) V – ; *43, Dactylis glomerata* L. (5) V + ; *19, Deschampsia caespitosa* Beauv. (5) V – ; *25, Glyceria aquatica* Wahl. (5) V – ; *157, Holcus lanatus* L. (5) V – ; *122, Hordeum murinum* L. (5); *126, Lolium perenne* L. (0); *199, Phalaris arundinacea* L. (0); *165, Phleum* (*? praecox*) (0); *39, P. pratense* L. (0) V – ; *213, Poa* sp. (0).

GENTIANACEAE. *85, Erythraea Centaurium* Druce (5) V – ; *232, Gentiana Amarella* L. (5).

HALORAGACEAE. *247, Hippuris vulgaris* L. (0).

HYPERICACEAE. *207, Hypericum Androsaemum* L. (0) V – ; *27, H. perforatum* L. (5) V – .

IRIDACEAE. *127, Montbretia* sp. (garden variety) (0).

LABIATEAE. *151, Ballota nigra* L. (0); *14, Clinopodium vulgare* L. (5) V – ; *21, Galeopsis Tetrahit* L. (0) V – ; *90, Lamium album* L. (5) V – ; *124, Lavandula officinalis* Chaix. (0); *35, Melissa officinalis* L. (0) V – ; *36a, Mentha* sp. (0) V – ; *36b, M. aquatica* L. (5) V – ; *45, Prunella vulgaris* L. (5) V – ; *51, Scutellaria galericulata* L. (0) V – ; *60, Stachys officinalis* Fr. (5) V – ; *61, S. sylvatica* L. (0) V – ; *68, Teucrium Scorodonia* L. (5) V – ; *102, Thymus* sp. (garden lemon thyme) (5) V – ; *292, T. serpyllum* L. (5).

LEGUMINOSEAE. *91, Lotus corniculatus* L. (5) V – ; *234, Medicago sativa* L. (5); *92, Melilotus officinalis* Lam. (0) V – ; *168, Ononis arvensis* L. (0); *246, Robinia hispida* L. (0); *288, Robinia pseudacacia* L. (10); *155, Trifolium repens* L. (0); *164, Trifolium* sp. (5); *245, Ulex europœus* L. (5); *72, Vicia Cracca* L. (5) V – ; *106, Wistaria sinensis* Sweet. (5) V – .

LILIACEAE. *135, Allium oleraceum* L. (0); *76, Asparagus officinalis* L. (0); *114, Convallaria majalis* L. (5) V – ; *129, Polygonatum* sp. (garden variety). (0); *276, Scilla nutans* (garden variety) (5) V + .

LINACEAE. *237, Linum catharticum* L. (0) V – .

LOGANIACEAE. *201, Buddleia* sp. (0).

LYTHRACEAE. *230, Lythrum Salicaria* L. (0).

MALVACEAE. *183, Althea rosea* (0); *222, Hibiscus syriacus* L. (0) V – ; *142, Malva sylvestris* L. (5).

MYRICACEAE. *287, Myrica Gale* L. (30).

NYMPHACEAE. *243, Nymphaea lutea* L. (0).

OLEACEAE. *214, Forsythia* sp. (0); *209, Jasminum nudiflorum* Lindley (0) V – ; *163, Ligustrum vulgare* L. (0).

Onagraceae. *194, Clarkia pulchella* (0); *113, Circaea lutetiana* L. (5) V – ; *84, Epilobium angustifolium* L. (5) V + ; *20, E. hirsutum* L. (5) V + ; *257, E. parviflorum* Schreb. (5); *218, Fuchsia* sp. (hardy garden variety) (10) V + ; *262, Oenothera biennis* L. (5) V – ; *277, Singhara* seeds (5) V –.

Oxalidaceae. *38, Oxalis Acetosella* L. (0) V –.

Papaveraceae. *205, Chelidonium majus* L. (5); *24, Glaucium flavum* Cr. (0) V – ; *227, Papaver Rhoeas* L. (5); *154, P. somniferum* L. (0).

Pinaceae. *263, Cedrus atlantica* Manetti (0).

Plantaginaceae. *40, Plantago media* L. (5) V –.

Plumbaginaceae. *261, Ceratiostigma* sp. (0); *241, Statice* sp. (annual garden variety) (5); *272, S. maritima* Mill. (15) V – ; *274, S. armeria* (large leaved garden) (5) V – ; *63, S. Limonium* L. (5); *62, Statice* sp. (perennial garden) (5) V –.

Polygonaceae. *283,* Buckwheat seed (of commerce) (5); *43, Polygonum amphibium* L. (5) V + ; *94, P. aviculare* L. (5) V – ; *221, P. cuspidatum* (10) V – ; *231, P. Hydropiper* L. (5); *235, P. Persicaria* L. (0); *130, Rumex acetosella* L. (0) V – ; *144, Rumex* sp. (10) V – ; *282,* Rhubarb root (of commerce). (50).

Polypodiaceae. *9, Dryopteris Filix-mas* Schott. (100) V +.

Polyporaceae. *136, Boletus* sp. (0); *173, Polystictus versicolor* (5); *174, Daedalea quercina* P. (0).

Primulaceae. *7, Anagallis arvensis* L. (0) V – ; *31, Lysimachia nemorum* L. (10) V – ; *32, L. Nummularia* L. (100) V + ; *240, L. vulgaris* L. (100) V +.

Punicaceae. *281, Punica granatum* (Root bark of commerce) (0).

Ranunculaceae. *108, Anemone japonica* (5) V – ; *47, Ranunculus sceleratus* L. (0) V – ; *101, Thalictrum minus* L. (5) V –.

Resedaceae. *258, Reseda Luteola* L. (0); *181, R. odorata* (0).

Rosaceae. *3, Agrimonia Eupatoria* L. (10) V – ; *4, Alchemilla vulgaris* L. (0) V – ; *166, Cretaegus Oxyacantha* L. (10); *116, Cydonia* sp. (garden variety) (0); *208, Malus* sp. (garden variety) (0); *95, Potentilla Anserina* L. (5) V + ; *186, P. reptans* L. (0); *150, Prunus spinosa* L. (0); *176, Pyracantha lelandii* (10) V – ; *49, Sanguisorba officinalis* L. (0) V – ; *59, Spirea Ulmaria* L. (5) V –.

Rubiaceae. *88, Galium Cruciata* Scop. (5) V – ; *22, G. verum* L. (5) V –.

Salicaceae. *256, Salix fragilis* L. (0).

Saxifragaceae. *251, Escallonia langleyense* (0); *206, Escallonia* sp. (5) V – ; *269, Hydrangea* sp. (0) V – ; *273, Philadelphus* sp. (5) V – ; *250, Saxifraga ligulata* Wall. (450) V + ; *270, S. umbrosa* L. (5) V –.

Scrophulariaceae. *78, Bartsia Odonites* Huds. (0) V – ; *118, Digitalis purpurea* L. (0); *87, Euphrasia officinalis* L. (5) V – ; *125, Linaria minor* Desf. (5); *29, L. vulgaris* Mill. (5) V – ; *204, Scrophularia aquatica* L. (0); *161, S. nodosa* L. (0); *71, Veronica Beccabunga* L. (0) V – ; *156, Verbascum Thapsus* L. (0).

Solanaceae. *89, Hyoscyamus niger L.* (5) V – ; *30, Lycium chinense* Mill (?); *182, Nicotiana tabacum* (0); *134, Solanum Dulcamara* L. (0); *99, S. lycopersicum.* (0) V – ; *236, S. nigrum* L. (0).

Thymeleaceae. *249, Daphne Mezereum* L. (0).

Tropaeolaceae. *248, Tropaeolum majus* (0).

Typhaceae. *58, Sparganium ramosum* Huds. (5) V –.

Umbelliferae. *8, Angelica sylvestris* L. (0) V – ; *146, Apium nodiflorum* R. f. (0); *16, Conium maculatum* L. (5) V – ; *18, Daucus carota* L. (5); *121, Foeniculum vulgare* Mill (0); *26, Heracleum Sphondylium* L. (0) V – ; *128, Myrrhis Odorata* Scop. (0); *37, Oenanthe fistulosa* L. (5) V – ; *93, Pastinaca sativa* L. (5) V – ; *41, Pimpinella major* Huds. (0) V – ; *42, P. Saxifraga* (5) V – ; *198, Sanicula europaea* L. (0) V – ; *54, Silaus flavescens* Bernh. (5) V – ; *56, Sison Amomum* L. (5) V – ; *70, Torilis anthriscus* Gmel. (5) V –.

Urticaceae. *197, Parietaria ramiflora* Moench. (0); *253, Urtica dioica* L. (0).

Violaceae. *105, Viola odorata* L. (?) V – ; *212, V. tricolor* L. (?) V –.

Thanks are due to Sir Jack Drummond, F.R.S., for his interest in this work and to Miss J. Sudworth for technical assistance. We are indebted to a number of workers, particularly Miss E. M. Gregory, for help in the collection of plants and to Dr R. Michaelis for extraction of the tannins.

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The Role of Aneurin in the Nutrition of *Tetrahymena geleii*

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SUMMARY: When fat and acetate are the principal substrates, the aneurin requirement of the ciliate *Tetrahymena geleii* is decreased but not entirely eliminated, in a way comparable to the aneurin-sparing effect of fats in metazoa. Aneurin-deficient cultures utilizing glucose or fat as substrates accumulate pyruvic acid.

Recent studies of intermediary metabolism emphasize the indispensability of carboxylation-decarboxylation reactions in mediating changes in length of carbon chain in a number of key compounds of the metabolic pool (Ochoa, 1951). That aneurin is needed for at least one such reaction—the decarboxylation of pyruvate—can be inferred from the observations that aneurin-deficient organisms accumulate pyruvate (birds and mammals, reviewed by Jansen, 1949; insects, Bhagvat & Sarma, 1943; the colourless alga *Prototheca*, Anderson, 1945). It is generally accepted that animals on a high-fat, low-carbohydrate diet are less dependent on aneurin—the so-called 'sparing action of fat'—but the extent of aneurin-sparing is highly uncertain (Robinson, 1951). Difficulties in devising an aneurin-free high-fat carbohydrate-free diet, and complications introduced by intestinal synthesis of aneurin, have hindered attempts to define the extent to which animals retain a need for aneurin as the substrate is varied. The ciliate *Tetrahymena geleii* provides a means of examining this situation: it utilizes fat, carbohydrate and intact protein; and, as in metazoa, aneurin-deficient organisms accumulate an α-keto acid (Tittler, 1949). In the present study this product was identified as pyruvic acid, and a residual requirement for aneurin was demonstrated when fat was the principal substrate.

METHODS

Culture technique. Strain H of *T. geleii* was obtained from Prof. R. P. Hall of New York University. Stock cultures were maintained in upright screw-capped tubes containing various chemically-defined (save for protogen) liquid media. Protogen (Stokstad, Hoffman, Regan, Fordham & Jukes, 1949), 3000–5000 units/ml., was obtained through the courtesy of Dr T. H. Jukes of Lederle Laboratories. For experiments, 5 ml. portions of culture medium were distributed in 50 ml. Erlenmeyer flasks; this allowed adequate aeration. The general culture technique has been described (Hutner, 1950). Growth was measured in terms of optical density as determined with a Klett-Summerson photoelectric colorimeter provided with a green filter having maximum transmission at 540 mμ. (Elliott, 1949). In some experiments M/25 [2-amino-2-methylol-1,3-propanediol], adjusted to pH 7·2, was used as buffer. This compound was well tolerated. In the later experiments the medium of Dewey, Parks & Kidder (1950), slightly modified (Table 1), was employed. All cultures were grown in the dark at 25°. Growth was measured after cultures had reached their peak so as to allow sufficient time for acid accumulation; a total of 6–9 days was generally required.

Table 1. *Composition of basal medium. Quantities in mg. dissolved in distilled water to* 100 *ml. final volume after adjustment to* pH 7·0 *with* NaOH

	(mg.)		(mg.)		(mg.)
DL-Alanine	77·0	Ca Pantothenate	0·06	$MgSO_4.7H_2O$	14·00
L-Arginine HCl	60·2	Nicotinamide	0·06	$Fe(NH_4)_2(SO_4)_2.6H_2O$	6·25
L-Aspartic acid	85·7	Pyridoxine HCl	0·60	$MnCl_2.4H_2O$	0·125
Glycine	7·0	Pyridoxal HCl	0·06	$ZnCl_2$	0·0125
L-Glutamic acid	163·0	Pyridoxamine HCl	0·06	$CaCl_2.2H_2O$	3·00
L-Histidine.HCl	29·4	Riboflavin	0·06	$CuCl_2.2H_2O$	0·30
DL-Isoleucine	88·2	Pteroylglutamic acid	0·006	$FeCl_3.6H_2O$	0·075
L-Leucine	135·7	Aneurin HCl*	0·60	KH_2PO_4	17·5
L-Lysine.HCl	106·4	Biotin	0·0003	K_2HPO_4	17·5
DL-Methionine	47·6	Choline chloride	0·60	Uracil	5·0
DL-Phenylalanine	70·0	Guanylic acid	15·0	Glucose†	500·0
L-Proline	122·5	Adenylic acid	10·0	Na acetate (anhyd.)	200·0
DL-Serine	108·0	Cytidylic acid	12·5	Tween 80‡	2400·0
DL-Threonine	123·0				
L-Tryptophan	16·8				
DL-Valine	92·4				

* Aneurin omitted in appropriate experiments.

† Autoclaved and added separately as a 50 % (w/v) solution. Protogen 2 units/ml.

‡ Polyoxyethylene sorbitan monooleate, Atlas Powder Co.

Determination of pyruvic acid. Pyruvic acid was estimated qualitatively according to Friedemann & Haugen (1943); quantitative determinations followed the colorimetric method described by Lardy (1949). In constructing reference curves, equivalent quantities of α-ketoglutaric acid were found to respond to the test. Consequently, Straub's (1936) more specific but less sensitive method, as described by Green, Leloir & Nocito (1945), was adopted; it is based on the formation of colour (absorption maximum at 440 mμ.) when pyruvic acid is condensed with salicylaldehyde in strongly alkaline solution. In agreement with Green *et al.* (1945), neither α-ketoglutarate nor oxaloacetate were found to interfere.

RESULTS

In early experiments where crude liver extracts were used as the source of protogen, aneurin was inactivated with little loss of protogen by treatment with sulphite (Williams, Waterman, Keresztesy & Buchman, 1935; Mason & Williams, 1942). Clear-cut aneurin deficiencies were also obtained when the liver extracts were replaced by purified protogen; sulphite treatment was then unnecessary.

Table 2 records the densities attained by cultures with increasing concentrations of aneurin in the modified medium of Dewey *et al.* (1950). The pH values indicate that acid accumulation is a function of: (*a*) total growth; (*b*) the

Table 2. *Influence of aneurin concentrations and presence of glucose on growth and acid production*

Aneurin (mμg./ml.)	Optical density		Final pH	
	No glucose	Glucose 0·05 % (w/v)	No glucose	Glucose 0·5 % (w/v)
0	0·42	0·38	7·9	7·1
1·0	0·43	0·45	8·0	6·1
10·0	0·47	0·70	8·0	4·5
50·0	0·50	0·86	8·2	7·0
100·0	0·64	0·91	8·3	8·0

concentration of aneurin; (*c*) the type of substrate. The appreciable growth in cultures containing neither added aneurin nor glucose suggested that the organism might use as substrate a constituent of the basal medium whose metabolism required little aneurin. Such a constituent proved to be Tween 80. Carbohydrate-free media containing Tween 80 together with 1·0 μg. aneurin/ml. supported as dense growth as did glucose media.

In the absence of information regarding a possible contamination of the protogen preparation by aneurin, it does not appear profitable to discuss the reason for the slight but definite growth in the absence of added aneurin. As purer protogen preparations become available, a precise answer to this question may be forthcoming.

The experiment outlined in Table 3, and several similar experiments, showed that acid production sharply decreased as the aneurin concentration was increased. In the main, the amount of pyruvate which accumulated paralleled the severity of aneurin deficiency, subject to the qualification that a minimal amount of aneurin (*c.* 10 mμg./ml.) had to be supplied to allow enough growth for detectable acid formation.

Table 3. *Acid production in cultures in the presence of* 0·5 % *glucose harvested at* 8 *days, as a function of aneurin concentration*

Aneurin added (mμg./ml.)	mm. Pyruvate/ml. medium	
	Straub method	Lardy method
10	10·0	11·8
100·0	0·85	0·47

The question arises as to whether aneurin-deficient organisms are killed by hydrogen ions or pyruvic acid, or, less directly, because of the limitation in available energy and building blocks resulting from the diminished ability to metabolize pyruvate. Experiments with 2-amino-2-methylol-1,3-propanediol buffer made it probable that death is caused by inability to utilize substrate, since the pH did not drop and growth and motility still depended on the presence of the same concentration of aneurin as required in unbuffered media.

The following compounds, tested individually, did not support growth in concentrations between 0·05 and 0·4 % (w/v); glucose served as standard:

Na acetate; Na_2 succinate. $6H_20$; NaH glutamate; DL-aspartate; Na_2 fumarate; D-mannitol; D-sorbitol; glycerol; sucrose; gluconate (neutralized free acid). Little weight is to be attached to negative experiments of this type, particularly with the acids, because of the familiar uncertainty in knowing whether the compounds had penetrated the cells.

DISCUSSION

The indispensability of aneurin for ciliates has been a troublesome question. In the past, media allowing a quantitative demonstration of the need for other vitamins, e.g. biotin and pteroylglutamic acid, have not yielded clear results for aneurin (cf. Kidder & Dewey, 1949). The demonstration of a residual aneurin requirement in the present experiments contrasts with the difficulties encountered elsewhere. This difference in results remains to be explained. One of the obvious potential sources of aneurin contamination is the protogen preparation; we may have been fortunate in having ours potent and yet virtually aneurin-free.

One might predict that organisms fed on 2-carbon compounds such as acetate, or materials yielding 2-carbon compounds, such as fat, would exhibit a lowered requirement for aneurin. They would have at their disposal the pre-formed building blocks for many cellular constituents, i.e. fat, steroids, and porphyrins. Starting from carbohydrate, the organisms would have first to make 2-carbon compounds via the aneurin-catalysed decarboxylation of pyruvate. It is conceivable that by simultaneously supplying a sufficient variety of substrates, in proper proportion, it might be possible to bypass other carboxylation reactions in which aneurin is concerned, thus further decreasing aneurin requirement. It appears from these experiments that *T. geleii* is a useful guide for elucidating problems of aneurin metabolism in metazoa and other organisms.

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The Effect of Phenol, 2-Phenoxyethanol and Cetyltrimethylammonium Bromide on the Oxidation of various Substrates by *Escherichia coli*

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SUMMARY: Effects of phenol, 2-phenoxyethanol and cetyltrimethylammonium bromide on the oxygen uptake of suspensions of *Escherichia coli* supplied with various single oxidizable substrates were examined. Phenol and 2-phenoxyethanol at low concentrations caused a stimulation of oxygen uptake in the presence of mannitol, glucose or lactose which was not associated with an increase in viable count, no stimulation with glycerol, and a retarded oxygen uptake with lactate, succinate or pyruvate. At higher concentrations phenol and 2-phenoxyethanol caused a retardation of oxygen uptake with all substrates, the degree varying with the substrate. Cetyltrimethylammonium bromide was toxic at very low concentrations and no stimulation of oxygen uptake by low concentrations or differential response in presence of different substrates was found.

Phenol, 2-phenoxyethanol (phenoxetol) and cetyltrimethylammonium bromide (cetrimide) chosen as representative of anionic, non-ionizing and cationic antiseptics respectively, were examined for their effect on respiratory mechanisms of *Esch. coli* as shown by the effect on oxygen uptake in presence of various substrates.

EXPERIMENTAL

Organism. The organism used was *Esch. coli* NCTC 5934. It was subcultured in nutrient broth, and freeze-dried (Proom & Hemmons, 1949). *Culture medium.* Nutrient broth was prepared by the tryptic digestion of lean meat; after autoclaving it had a pH of 7·2–7·4. It was solidified when required by the addition of 2 % agar.

Chemicals. The 2-phenoxyethanol (phenoxetol) was supplied by Nipa Laboratories Ltd., Cardiff. The cetyltrimethylammonium bromide (cetrimide) was a commercial sample which, assayed by the method of The British Pharmaceutical Codex (1949), contained 84·4 % (w/w) of $C_{19}H_{33}(CH_3)_3NBr$. The sugars and mannitol used were of bacteriological quality as supplied by Messrs T. Kerfoot Ltd.; sodium pyruvate was prepared by the method of Robertson (1942); all other chemicals were of A.R. quality.

Phosphate buffers. 0·1 M or 0·05 M-KH_2PO_4 and $Na_2HPO_4.12H_2O$ were mixed immediately before use, to give a pH value of 7·0 (electrometric). *Ringer's solution* was prepared according to Wilson (1922) and sterilized by autoclaving; final pH *c.* 7·5.

Bacterial suspensions. The contents of a tube of freeze-dried material were reconstituted with nutrient broth, grown at 37° for 18 hr., then subcultured twice and inoculated on to nutrient agar. After 24 hr. at 37° this slope was stored at room temperature and served as the immediate source of organism during 3–4 weeks, after which a new slope was made from another freeze-dried tube. This method yielded organisms giving reproducible results. For the preparation of suspensions, a third subculture from the agar slope, grown in 5 ml. nutrient broth for 18 hr. at 37°, was used to inoculate 250 ml. nutrient agar contained in a 3 l. Thomson culture bottle. This culture was incubated at 37° for 48 hr., the cells washed off with the Ringer's solution, the suspension centrifuged twice and resuspended in Ringer's solution. Suspensions so prepared were standardized by means of a photoelectric nephelometer to contain 6·2–6·8 mg. dry weight cells/ml. and stored at 4°. Under these conditions suspensions retained their activity for at least 8 days.

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Determination of oxygen uptake. The usual Warburg technique was used. Usually the main compartment of the Warburg vessel contained 1 ml. substrate (0·02 M) in phosphate buffer (0·05 M) and 0·5 ml. solution of the antiseptic; in side arm, 0·5 ml. bacterial suspension; in centre well 0·2 ml. 20 % NaOH solution and an 18 mm. square of folded filter paper. To study higher concentrations of the relatively insoluble phenoxetol, the main vessel contained 0·5 ml. of 0·04 M substrate in 0·1 M phosphate buffer and 1 ml. of antiseptic solution. The gas phase was air; all measurements were made at 37° and 100 oscillations/min.

The effects of the three antiseptics on the oxidations of mannitol, glucose, lactose, glycerol, pyruvate, lactate and succinate were studied, at substrate concentration 0·01 M to ensure excess. With zero and low concentrations of antiseptics the relationship between time and oxygen uptake was linear, except with lactose, when the rate increased during the first 60 min. The rate of oxidation was proportional to the volume of bacterial suspension used. In Figs. 1–4 the oxygen uptake values in presence of antiseptic are expressed as percentages of the uptake in the absence of antiseptic at the time stated. The activity of the suspensions under the conditions adopted can be judged from the following Q_{O_2} values (μl. O_2/mg. dry weight cells/hr.) for various substrates: succinate (114), glucose (100), lactate (88), pyruvate (82), glycerol (80), mannitol (72), lactose (48).

Viable counts. Volumes of substrate solution, antiseptic solution and cell suspension as used in the Warburg flasks were placed in 25 ml. conical flasks and incubated at 37°. At the end of a period of time equal to that of the corresponding Warburg experiment a 1 ml. sample was removed and diluted serially in Ringer solution to give 100–200 colonies/plate on nutrient agar. Counts were made after 3 days' incubation at 37°. When cetrimide was the inhibitor, the procedure was modified in that the first ml. of inoculum was placed in 9 ml. of sterile 1 % Lubrol W. (Imperial Chemical Industries Ltd.), in a 1 oz. McCartney bottle containing 18–20 glass beads (3 mm. diam.), and well shaken to disperse clumped organisms before further dilution in Ringer's solution and plating (Davies, 1949).

RESULTS

Each experiment involved a study of the time course of oxygen uptake in presence of graded doses of antiseptic and a single substrate. From these observations two types of curve were constructed: (*a*) time-graded action curves where, for each antiseptic concentration, the oxygen uptake was plotted against time; (*b*) dose-response curves where dose of antiseptic was plotted against volume of oxygen utilized at the end of a given period.

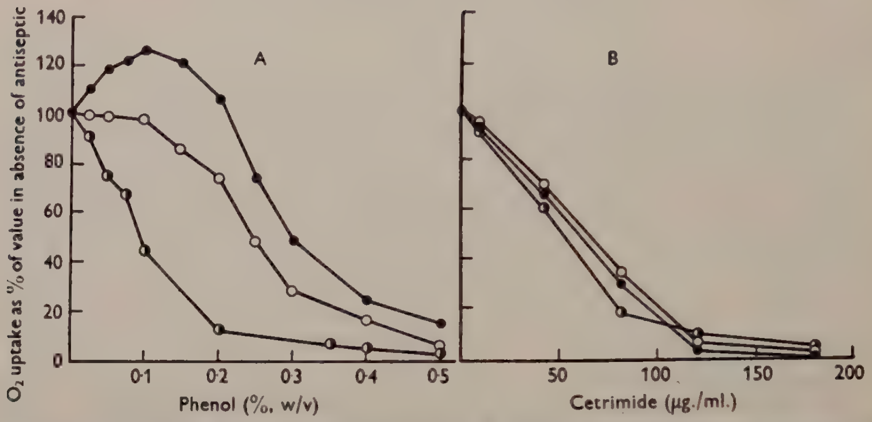

Fig. 1. Dose-response curves. Oxygen uptake in 60 min. against various concentration of phenol (A) and cetrimide (B). Suspensions of *Esch. coli* at pH 7·0, 37°. Substrates: ●—●, mannitol; ◑—◑, = succinate; ○—○, = glycerol. Substrate concentrations 0·01 M.

The dose-response curves with phenol and phenoxetol were of three types, according to the substrate being oxidized (an example of each type is shown in Fig. 1A). *Curves obtained with mannitol, glucose or lactose as substrate.* A stimulation in the rate of oxygen uptake occurred with phenol at concentrations up to 0·2 % (w/v) and with phenoxetol up to 0·3 % (w/v); the rate of oxygen uptake was increasingly depressed by the higher concentrations of antiseptic. *Curves obtained with glycerol as substrate.* The rate of oxygen uptake was not affected by concentrations of phenol below 0·15 % (w/v) or of phenoxetol below 0·25 % (w/v), but was progressively depressed by higher concentrations. *Curves obtained with succinate, lactate or pyruvate as substrate.* Even the lowest concentrations tested (0·025 %, w/v, for phenol and 0·1 %, w/v, for phenoxetol) caused decreased oxygen uptakes, progressively more marked at higher concentrations.

With cetrimide, differences of response with different substrates were not seen and no stimulation of oxygen uptake was observed (Fig. 1B).

Stimulation or inhibition of oxygen uptake, when it occurred, was noticeable within 5 min. of application of the antiseptic and, except at the higher concentrations, this rate which was characteristic for each substrate-antiseptic combination was maintained for the duration of the experiments. Viable counts showed no increase in the number of organisms over the stimulating dose range. The form of the curves relating viable count at the end of 60 min. to dose of drug showed that there was some parallelism, with each of the three antiseptics used, between decrease in oxygen uptake with increasing dose of

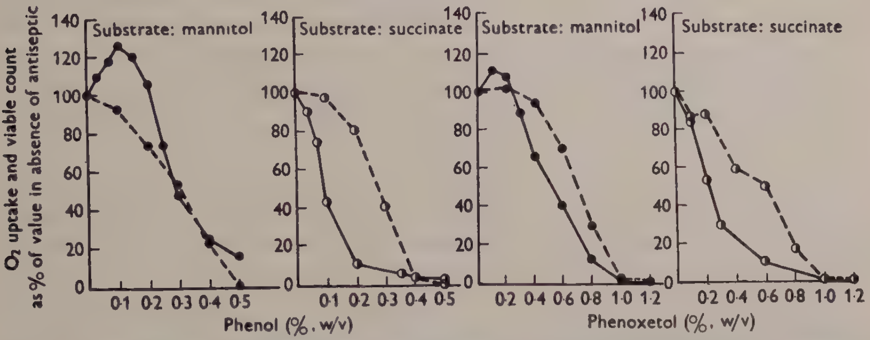

Fig. 2. Oxygen uptake in 60 min. and viable count at end of 60 min. at various concentrations of phenol and phenoxetol. pH 7·0, temp. 37°. ——, oxygen uptake; ----, viable count.

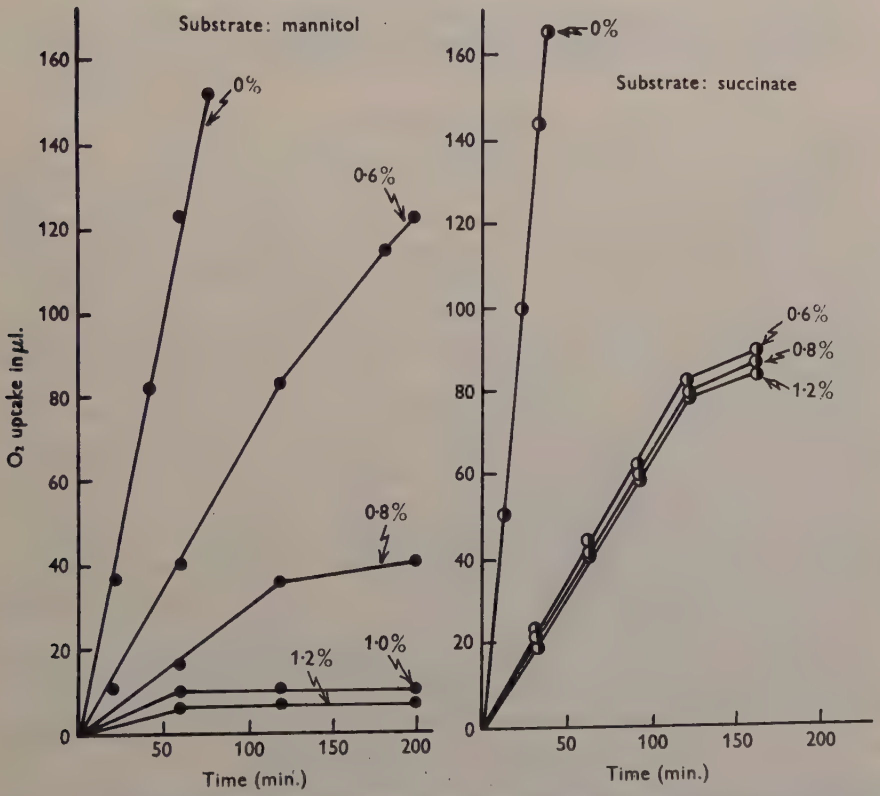

Fig. 3. Time-graded action curves showing the effect of high concentrations of phenoxetol on the course of oxygen uptake in presence of mannitol and succinate. Numbers on curves indicate % (w/v) phenoxetol.

antiseptic and the decrease in the viable population (results with phenol and phenoxetol are shown in Fig. 2).

The nature of the substrate modified the response to higher concentrations of phenol and phenoxetol. With mannitol and glycerol a concentration of 0·6 % (w/v) phenoxetol caused *c.* 70 % decrease in rate of oxidation, and 1·0 % phenoxetol completely abolished respiratory activity after 60 min. (Fig. 3). With succinate and lactate there was no significant difference between the effects of 0·6 and 1·2 % (w/v) phenoxetol, during 150 min., when a steady but very gradually decreasing oxygen uptake was observed (Fig. 3).

Antiseptics and detergents, under certain conditions promote the leakage of cell constituents from bacteria (Hotchkiss, 1946; Gale & Taylor, 1947; Salton, 1950, 1951) and enable sugars to enter cells more readily (Deere, 1939). Evidence has also been obtained that the enzymes concerned with the utilization of certain sugars are intracellular (Deere, 1939), whereas those concerned in the oxidation of organic acids may be at or near the cell surface (Quastel, 1926). The results obtained here with phenol and phenoxetol may be related to their effect on substrate permeability, and on access to the oxidizing enzymes.

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Staphylococcal Coagulase: Mode of Action and Antigenicity

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SUMMARY: Different strains of staphylococci vary in ability to produce free coagulase. Methods are given for the selection of suitable strains and for the production and concentration of coagulase from one of them. Of animal plasmas examined, cow, sheep, dog, guinea-pig and mouse showed a relative deficiency in coagulase activator, while plasma of man, monkey, horse, cat, pig, fowl and rabbit contained the most.

Coagulase activator resembles prothrombin closely in physical properties such as thermolability, behaviour during salting out and removal by adsorbents. They are both partly lost in blood clotting and in dicumarol poisoning, but while prothrombin is apparently removed from plasma by repeated Seitz filtration there is no loss of activator. The question of probable identity is discussed.

Antibody to coagulase is present in the sera of many normal individuals and in those suffering from chronic staphylococcal infections. The intramuscular injection of coagulase adsorbed on aluminium phosphate produces similar antibodies in the sera of rabbits.

The marked association between the pathogenicity of staphylococci and their ability to coagulate the plasmas of certain animals has led to a more extensive study of the properties and mode of action of the clotting agent, staphylococcal coagulase, first described by Loeb in 1903. Two findings in recent years are of especial importance. First, the discovery of Smith & Hale (1944) that coagulase brought about the clotting of fibrinogen through the agency of a third substance present in the plasma and tissues, and variously termed coagulase activator (Smith & Hale, 1944), coagulase reacting factor (Tager, 1948*a*) or coagulase globulin (Miale, 1949). Secondly, the finding that, contrary to earlier observations (Gross, 1931; Walston, 1935; Smith & Hale, 1944), antibodies against coagulase can in fact be produced (Tager & Hales, 1948*c*; Rammelkamp, Hezebicks & Dingle, 1950).

The work now reported was begun in 1948 with a view to finding whether coagulase was antigenic and if so, how the antigen could be best prepared and administered. During this work various observations were published which tended either to confirm or deny our findings, especially those regarding the nature of coagulase activator. The results now reported may throw some light on these observations, but are preliminary to further studies on the use of coagulase antigen in the production of immunity to staphylococcal infections.

MATERIALS AND METHODS

Assay of coagulase. Many workers (Lominski & Roberts, 1946; Tager & Hales, 1947; Kaplan & Spink, 1948) measure coagulase by determining the lowest concentration which clots human plasma, usually diluted, after a given period of incubation at 37°. More recently, Rammelkamp, Badger, Dingle, Feller & Hodges (1949), used as the clottable agent a solution of bovine fibrinogen to which a minute amount of human serum was added as activator. In the present work, 0·2 ml. volumes of falling dilutions of coagulase in saline buffered to pH 7·4 were added to 0·2 ml. volumes of 0·1 % human fibrinogen (ether precipitated and dried) containing 5 % rabbit plasma as activator, in tubes measuring 5·0 × 0·8 cm. This amount of rabbit plasma was nearly optimal for the reaction, so that the different amounts of activator present in different batches of fibrinogen could be neglected. The rabbit plasma was stored for months at −20° without any loss of activity, and the fibrinogen plasma mixture was likewise stable for a few hours when frozen. The tubes were incubated at 37° for 30 min. or 1 hr.; 30 min. was adequate for most purposes. Since the reaction proceeds stoicheiometrically the titre at 1 hr. is double that at 30 min. (Tager, 1948*b*). At the end of 30 or 60 min., as the case may be, one drop (0·02 ml.) of a 1 % aqueous suspension of Supercel (Johns Manville) was added to each tube, and after dispersing the particles of Supercel the rack of tubes was placed in the refrigerator for 20–30 min. Falling dilutions of a standard preparation of coagulase were included in each test, and after further shaking these were matched visually with the unknown. The Supercel particles were found in different degrees of dispersion depending on the amount of coagulation which had occurred in each tube, and differences in flocculation corresponding to ± 10 % in the coagulase concentration could be recognized. The M.C.D. or minimal clotting dose was defined as that amount of coagulase/ml. which, when mixed with 1 ml. plasma fibrinogen and incubated for 1 hr. at 37° under the conditions specified, gave just visible flocculation of the particles. Most batches prepared with the 'Newman' strain of *Staphylococcus aureus* had values of 3000–3500 M.C.D./ml.

Assay of coagulase activator; method A. In the early stages of the work 0·2 ml. volumes of falling dilutions of the solution to be assayed were added to 0·8 ml. volumes of buffered saline (pH 7·4) containing 0·2 % (w/v) of bovine fibrinogen (ether precipitated and dried). These mixtures were held at 37°, and 0·2 ml. of coagulase solution was then added, the time of clotting being measured from the time of addition of coagulase. Reference to a curve prepared by diluting a known solution of activator allowed comparisons to be made with the activator content of the solution tested.

Assay of coagulase activator: method B. More recently it has been found possible to measure minimal amounts of activator by adding falling dilutions to tubes containing coagulase and bovine fibrinogen, the former in concentrations insufficient to clot the fibrinogen in the presence of the small amount of bovine activator present in the particular sample. Since a deficiency of activator is compensated for to some extent by an excess of coagulase, traces of activator were easily detected, but for most purposes submaximal amounts of coagulase were sufficient. In practice 0·2 ml. volumes of falling dilutions of activator in buffer saline were added to 0·2 ml. volumes of 0·4 % bovine fibrinogen, followed by 0·2 ml. coagulase solution containing as a maximum 3,000 M.C.D./ml. After incubation for 1 hr. at 37°, 0·02 ml. of Supercel suspension (1 %) was added and the tubes read as in the coagulase assay test.

Assay of antibody. Because of the instability of coagulase at alkaline pH values, all dilutions were made in a mixture of 1 vol. M/15 phosphate buffer (pH 7·0) and 2 vol. 0·85 % saline. The stock solution of coagulase was the partially purified cadmium precipitated material described later, dissolved at 5 mg./10 ml. of 0·01 M-HCl. This solution was diluted immediately before use in buffer saline to give 20 M.C.D./ml. Volumes (0·1 ml.) of falling dilutions of serum were mixed with equal volumes of the diluted coagulase and were left for 30 min. at room temperature, after which 0·2 ml. of 0·1 % (w/v) fibrinogen containing 5·0 % (v/v) rabbit plasma was added to each tube and the mixtures incubated at 37° for 1 hr. Supercel suspension (1 %) was added and the tubes read after 30 min. at 4°. The reciprocal of the serum dilution just sufficient to allow minimal clotting was taken as the titre of the serum expressed in units. A serum of known value was included in all tests for comparison. The sera were not inactivated but were incubated with heparin for a short time to destroy thrombin and were stored frozen if not used immediately. The stability of the coagulase at pH 7·0 is less than at pH 2·0, but there was no demonstrable loss when equal volumes of coagulase (20 M.C.D./ml.) were mixed with either saline or normal rabbit serum diluted 1/2 and allowed to stand for 30 min. at room temperature as in the assay, provided that the final pH value of the mixture was 7·0.

EXPERIMENTAL

Strain selection and production of coagulase

The coagulase used in these experiments was produced by a strain of *Staph. aureus*, 'Newman' (NCTC 8178), originally isolated from a case of secondarily infected tubercular osteomyelitis in man. It was the best free-coagulase producer among a large number of staphylococci isolated from human and animal sources; the stability of its coagulase was greater than that of other strains tested. This strain was selected by choosing colonies on nutrient agar plates containing 50 mg./human fibrinogen (ether precipitated) and 2·5 ml. human plasma/100 ml. medium, these components being added to the molten agar immediately before pouring and at a temperature not exceeding 50°. After 18 hr. at 37° suitable colonies were surrounded by a zone of fibrin 2–3 mm. in diameter, which increased in size when the plates were left at room temperature. Many strains showed partial clearing of this zone through the action of either a protease or of staphylokinase, but this did not occur with the 'Newman' strain. Selected colonies were subcultured into 25 ml. conical flasks containing about 5 ml. nutrient broth. These cultures were grown for about 12 hr. at rest and were then agitated for several hours at 37° until full growth had been reached. The whole cultures and the centrifuged supernatants were then tested for coagulase activity. In the majority of strains the coagulase was about equally divided between cells and supernatant fluid, but in the 'Newman' strain some 90 % of it was in the supernatant. Since the 'Newman' strain was in all respects the most suitable for this study, all work henceforth described was done with this strain. It must be emphasized that later work has shown that coagulase from other strains may behave differently.

Using the strain 'Newman', any good digest or infusion broth gave optimal coagulase production at full growth, which could not be enhanced by the use of oxygen and carbon dioxide mixtures or by subsequent autolysis. The 'Casamino acid' medium used by Rogers (1945) gave equally good growth but poor coagulase production. Shaken cultures contained *c.* 4–5 times as much coagulase as did unshaken cultures, the amounts being roughly proportional to the amount of growth. For the production of large amounts of coagulase, 2 l. conical flasks containing 400 ml. broth were heavily seeded with a freshly grown culture and treated in the same manner as the smaller flasks. Since on subculture or storage in media the 'Newman' strain produced occasional variants which were relatively poor coagulase producers, it was important to pick, from time to time, good coagulase-producing colonies from fibrinogen plates.

Table 1. *The influence of temperature and* pH *on the stability of crude coagulase*

A culture filtrate of high coagulase content was used. The pH was altered by the addition of dilute HCl or dilute NaOH. After incubation, all samples were neutralized and tested for coagulase activity.

		pH value				
		2	**4**	**5**	**7**	**9**
Time (hr.)	**Temperature**	**Loss in coagulase activity (%)**				
2	37°	0	50	0	0	20
2	60°	20	90	95	60	70
1	100°	100	100	100	100	100

Concentration of coagulase

For sterilization and concentration advantage was taken of the marked stability of coagulase at pH 2 (Table 1). Bacteria were removed from the culture by mixing with 'Filter cel' and filtration through paper, after which the filtrate containing the coagulase was cooled and all subsequent manipulations carried out at 4°. Cadmium sulphate was added to a final concentration of 0·5% (w/v) and the mixture adjusted to pH 5·8. The flocculant precipitate was collected next day and dissolved by the gentle addition of N-HCl to a final pH value of *c.* 2·0. This solution was dialysed against running tap water at pH 2·0 until free from cadmium, insoluble material was removed, the preparation lyophil dried. The total loss was about 10 %.

Properties of the coagulase preparation

In agreement with Tager (1948*b*), we found that coagulase was rapidly destroyed by proteolytic enzymes; as little as 1 part of crystalline trypsin in 10^8 of solution was able to destroy 3000 M.C.D. in 4 hr. at 37°. Partially purified coagulase material had an activity of 6000 M.C.D./mg.; it contained *c.* 60 % protein, so that its activity was 10,000 M.C.D./mg. protein. Its most remarkable property was its stability at pH 2·0, there being little destruction in several hours at 56° when held at this pH. A solution of 1 mg./ml. had no action on rabbit cells or guinea-pig skin, and did not liquefy a fibrin clot in 24 hr. at 37°. It had slight phosphatase activity and 1 mg. destroyed 30 units of penicillin in 1 hr. at 37°.

Occurrence of coagulase activator

Assay method B enabled us to compare the concentration of coagulase activator present in the various plasmas. The animals so far examined fell into two well-marked groups: human, monkey, horse, cat, pig, fowl and rabbit plasmas showed clotting at final dilutions of 1/150,000 to 1/200,000, while cow, sheep, dog, guinea-pig and mouse plasmas reacted at final dilutions of 1/3,000 to 1/10,000. A comparison of the plasmas of a number of individual members of each species showed little or no variation from the figures given.

Comparison of coagulase activator and prothrombin

Since the first description of coagulase activator by Smith & Hale (1944), various authors (Tager & Hales, 1948*a*; Tager, 1948*b*; Kaplan & Spink, 1948) have studied the occurrence of activator in human and other plasmas, bearing in mind its possible identity with one of the known plasma components. Tager & Hales (1948*a*), using human plasma, noted a similarity in properties between activator and prothrombin, but because of certain discrepancies they did not conclude that the two were identical. The most important separation of activator and prothrombin appeared on repeated Seitz filtration of human plasma, the filtrate showing no loss of activator but failing to clot on re-calcification and addition of thromboplastin. Nevertheless, some prothrombin was left, since Tager & Hales found that acid precipitation of this filtrate, following dilution in distilled water according to Mellanby's method (1930), showed prothrombin to be present in small amounts in the globulin precipitate.

The question is further complicated by discrepancies arising out of the study of plasma fractions obtained by $(NH_4)_2SO_4$ precipitation and by ethanol precipitation in the cold at controlled ionic and H-ion concentrations (Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946). Results obtained by different authors are summarized below:

	Fraction containing coagulase activator after	
Author	$(NH_4)_2SO_4$ precipitation	Ethanol precipitation
Tager (1948*a*)	Globulin at 40–50 % saturation	Fractions III-1, IV-2, IV-1.
Kaplan & Spink (1948)	Albumin at 80–100 % saturation	Fractions IV-4 and little in V
Miale (1949)	Albumin at 100 % saturation	Fraction IV-4 and IV-1
Gerheim, Ferguson & Travis (1947)	Albumin	—

It is interesting that all the ethanol fractions which show activity are globulin fractions, although three of the above workers found that activator occurred with the albumin in $(NH_4)_2SO_4$ fractionation. From the work of Cohn *et al.* it is known that most of the prothrombin is in fraction IV-2. In our own studies prothrombin was measured by a one-stage technique similar to that of Quick (1942), factor V being added to the mixtures when necessary.

Comparison of plasma and serum. Before reading the papers of the above-mentioned authors our attention had been drawn to the possibility that coagulase activator was related to prothrombin by the observation that both human and rabbit plasma contained much more activator than did the corresponding sera, as shown by a comparison of the two at various dilutions (Table 2).

Table 2. *Comparison of activator content of rabbit serum and plasma*

Serial dilutions (in saline and buffer, pH 7·4) of serum and plasma from the same rabbit were tested against coagulase for the presence of activator, using method A.

	Clotting time			
Dilution	Serum		Heparinized plasma	
	min.	sec.	min.	sec.
1/1	2	52	2	40
1/2	3	54	2	40
1/4	5	58	2	38
1/8	7	40	2	40
1/16	11	30	2	20
1/32	14	45	2	35
1/64	19	10	2	45
1/128	25	—	3	15
1/256	31	—	3	58
1/512	45	—	4	38

Destruction by thromboplastin. In the experiment shown in Table 3, mixture A consisted of fresh plasma with brain thromboplastin and free calcium, while mixture B contained plasma and thromboplastin but no free calcium. After incubation the prothrombin concentrations in both mixtures were estimated by a modified one-stage technique, fibrinogen, thromboplastin and calcium being added in the quantities shown in the third column of Table 3. Activator content was measured by method B. As will be seen, mixture A, which contained free calcium, showed almost complete loss of coagulase and activator, but there was little or no loss in mixture B. In this experiment the free calcium both in mixture A and in the subsequent prothrombin estimations was between 2·5 and 3·5 mM., and in the prothrombin dilution curves from which the prothrombin loss was estimated both calcium and factor V levels were optimum. Nevertheless, the losses given are only approximations. A similar destruction of both prothrombin and activator occurred with Russell's viper venom.

Dicumarol. Rabbits poisoned with dicumarol showed a marked decrease in the activator and prothrombin contents of their plasma, the decreases being approximately the same; similar decreases were found in the plasmas of patients under dicumarol therapy.

Table 3. *Comparison of loss of coagulase activator and prothrombin in plasma incubated with thromboplastin in the presence of calcium ion (mixture A), and in the absence of calcium ion (mixture B). Plasma separated from 9 vol. human blood + 1 vol. 100 mM. potassium oxalate*

	Prothrombin test mixture	Prothrombin loss	Activator loss
Mixture A (Ca present)			
0·4 ml. plasma	0·1 ml. mixture A	—	—
0·2 ml. brain extract	0·1 ml. human fibrinogen (0·4 %)	About 99 %	About 96 %
0·2 ml. CaCl$_2$ 37 mM.	0·1 ml. brain extract	—	—
Incubated 1 hr., 37°	0·1 ml. 7 mM. CaCl$_2$	—	—
	Clotting time 4 min. 30 sec.	—	—
Mixture B (Ca absent)			
0·4 ml. plasma	0·1 ml. mixture B	—	—
0·2 ml. brain extract	0·1 ml. human fibrinogen (0·4 %)	—	—
0·2 ml. saline	0·1 ml. brain extract	Nil	Nil
Incubated 1 hr., 37°	0·1 ml. 20 mM.-CaCl$_2$	—	—
	Clotting time 20 sec.	—	—

$(NH_4)_2SO_4$ *fractionation.* A reinvestigation of the fractions obtained with ammonium sulphate confirmed the findings of Tager (1948*a*); all of the activator was found in the globulin fraction and was precipitated almost completely by 60 % saturation with ammonium sulphate. The prothrombin loss was apparently greater than that of the activator and probably occurred during the subsequent dialysis of the fractions against citrate-saline, a procedure not necessary for the measurement of activator.

Seitz filtration. Repeated Seitz filtration of plasma removed prothrombin without comparable loss of activator. In the cases of human plasma there was an apparent increase of the latter as noted by Tager & Hales (1948*a*). Like these authors, we found that although this filtered plasma was apparently free from prothrombin, the precipitate obtained when it was diluted with distilled water and precipitated at pH 5·0, when redissolved, clotted on addition of $CaCl_2$. If this were due to prothrombin in the filtrate, then its activity was less than 1 % of that present in the original plasma.*

Purification by adsorption. Like prothrombin, coagulase activator is readily adsorbed from plasma by barium sulphate or freshly prepared aluminium hydroxide; a specimen of dried purified prothrombin prepared by Dr Rosemary Biggs (by adsorption of fresh human plasma with Cα alumina followed by elution) was found to function well as coagulase activator at very high dilutions. This protein was used in the experiments shown in Table 4, in which the minimal amount of activator needed to produce flocculation of fibrinogen was measured for falling concentrations of coagulase using coagulase assay method B. Thus at 16,000 M.C.D. coagulase/ml. a final dilution of $1/10^8$ of purified prothrombin (measured as protein) was sufficient. Lower concentrations of coagulase required more activator, but no simple relationship was found between the coagulase and activator concentrations required, and their product increased as the coagulase concentrations fell.

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* The studies of Koller, Loeliger & Duckert (1951) now make it doubtful if Seitz filtered plasma is always free of prothrombin.

Table 4. *Concentration of activator required to produce minimal clotting for decreasing concentrations of coagulase (Method B)*

Coagulase concentration added (M.C.D./ml.)	Activator concentration added (mg. protein/ml.) ($\times 10^{-6}$)
16,000	0·03
8,000	0·06
4,000	0·2
2,000	0·5
1,000	2
500	20

Stability. We investigated the stability of activator and prothrombin in rabbit plasma kept at different pH values and temperatures (Table 5). There was marked loss of both components at the acid and alkaline values except at 60°, where both were most stable at the acid level. The 30 min. value at 60° is not given, since the loss was complete in all cases. In spite of possible errors due to the approximate nature of the measurements, there was surprisingly good agreement between the stability of the two substances in the range investigated.

Table 5. *The influence of heat at different* pH *values on rabbit activator and rabbit prothrombin using rabbit plasma (diluted* 1/4)

Factor V added in all prothrombin estimations. Activator measured by method A.

		Residual activator (%)			Residual prothrombin (%)		
Time (min.)	Temp.	pH 4	pH 7	pH 11	pH 4	pH 7	pH 11
30	25°	15	100	60	14	100	75
30	37°	3	100	8	5	100	4
10	60°	12	0·1	0·1	15	Nil	Nil

Coagulase clotting. Both Smith & Hale (1944), using testis extract as activator, and Miale (1949), using the albumin fraction of plasma, found that the incubation of coagulase and activator brought about the formation of a thrombin-like product, as shown by a progressive decrease in the clotting time when samples of the mixture were taken at intervals and added to fibrinogen. We found that this phenomenon is only apparent at certain dilutions of both coagulase and activator (Table 6), using as activator the purified prothrombin preparation previously mentioned. In this experiment prior incubation of 300 M.C.D. coagulase/ml. with all of the activator dilutions gave shorter clotting times than in the controls, but this was not found with the lower coagulase value. In a previous report (1950), based on experiments made with human plasma, we stated that coagulase was lost during clotting; this does not occur with rabbit plasma, and it is therefore likely that the loss we then found was due to the presence of an inhibitor in the human plasma used. With rabbit plasma we were likewise unable to demonstrate any loss in either prothrombin or activator during coagulase clotting, but it is possible that the loss is less than can be detected by the methods used. If coagulase activator is identical with prothrombin, then the thrombin-like product is not thrombin, since it is not inhibited by heparin.

Serum inhibition

A number of workers (Lominski & Roberts, 1946; Bekker, 1947; Tager & Hales, 1948*b*; Rammelkamp *et al.* 1950) have noted the presence of a coagulase inhibitor in the plasma of certain normal humans, while Kaplan & Spink (1948) and Gerheim & Ferguson (1949) found a coagulase inhibitor in the sera of other animals. Lominski & Roberts detected inhibition in just over 50 % of all human sera examined, while Tager & Hales found only 7 % of all human plasmas to be devoid of inhibitor.

Human sera. Since the proportion of sera from normal humans showing antibodies to coagulase will depend on the sensitivity of the method used, it was of interest to measure some normal levels by the present method. The sera of twenty-five members of the laboratory staff showed the following distribution of antibodies: six = nil; four = 0–1 unit/ml.; ten = –10 units/ml.; two = 10–20 units/ml.; two = 20–25 units/ml.; and one = 60 units/ml. In this small series there was no clear association between past staphylococcal infection and the presence of antibodies. Sera from cases of chronic deep-seated staphylococcal infections all showed high anticoagulase titres, 100 units/ml. being present in some cases.

Artificial immunization. Washed aluminium phosphate suspension (6 mg./ml.) prepared according to the method of Holt (1950) adsorbed up to 3·5 mg. coagulase/mg. aluminium phosphate at pH 4·8 from a 1 % solution of the partly purified coagulase. This material gave higher and more constant levels of anticoagulase in the sera of rabbits when injected either intramuscularly or subcutaneously, than did injections of coagulase alone, and was used in all the later experiments. The experiment shown in Table 7 was one of a series designed to find the optimal amounts of both aluminium phosphate and coagulase required. In this instance an increase in the amount of coagulase injected was not accompanied by an increase in the titre reached 14 days after the final injections. Nevertheless, all animals responded; four animals gave 5 units/ml. after one injection, and two reached 20 units/ml. 14 days after the third injection of 2 mg. coagulase. The highest titre so far reached was 40 units/ml. following 3 injections over 6 weeks of larger amounts of more impure coagulase.

DISCUSSION

The many conflicting reports in the literature on the properties of staphylococcal coagulase are in part due to the use of totally different strains of staphylococci grown on different media. Whereas it is usual to use coagulase which has been separated from the cells by filtration or centrifugation, some authors have used suspensions of living or of heat-killed organisms, and this in our experience will affect the results. Furthermore, as Tager (1948*b*) showed, a highly purified coagulase may exhibit qualities different from those of the

Table 6. *Decrease in clotting time following prior incubation of equal volumes of coagulase and purified activator (titre 1/60,000) at 37° for 30 min.*

Coagulase (M.C.D./ml.)	Incubated										Non-incubated control									
	1/100		1/500		1/1000		1/2000		1/4000		1/100		1/500		1/1000		1/2000		1/4000	
	Clotting time																			
	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.
300	18	—	18	30	22	45	42	45	78	—	20	30	25	—	41	40	51	30	93	—
75	39	40	51	35	47	40	78	—	129	—	33	40	41	30	78	—	78	—	—	—

Table 7. *Production of anticoagulase in rabbits using coagulase adsorbed on* $AlPO_4$. *Injections given on 1st, 15th and 29th days of experiment*

	Dosage (mg.)		Day 14				Day 28				Day 42					
			Serum dilution													
Rabbit	$AlPO_4$	Coagulase	1	2½	5	10	1	2½	5	10	1	2½	5	10	20	30
			Degree of clotting*													
19	3	2	−	−	±	+	−	−	−	+	−	−	−	−	+	++
20	3	2	−	±	+	++	−	−	−	±	−	−	−	−	±	+
21	3	4	−	−	±	+	−	−	±	+	−	−	+	++	++	++
22	3	4	++	++	++	++	−	±	++	++	−	+	++	++	++	++
23	3	8	−	−	+	++	−	−	+	++	−	−	−	+	++	++
24	3	8	−	−	±	+	−	−	±	+	−	−	−	+	++	++
Control	0	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++

* Full clotting = + + ; intermediate clot = + or ± ; no clot = − ; serum samples tested day before second and third injections and 13 days after last injection.

crude culture filtrate. We have now found other strains which produce large amounts of extracellular coagulase with properties somewhat different from the coagulase produced by the 'Newman' strain.

Our experiments indicate a similarity between the properties of coagulase activator and prothrombin; because of the role of the latter in normal clotting it is tempting to consider that they may be identical. We find that they are similar in physical behaviour; both are partly lost in normal clotting and on incubation with thromboplastin in the presence of Ca ion, and they both decrease in concentration almost *pari passu* in dicumarol poisoning. A similar decrease has recently been found in a case of obstructive jaundice, where both activator and prothrombin levels were diminished to 25 % of normal. The work of other workers with ethanol precipitated fractions not available to us shows a wider spread of activator than of prothrombin over the various fractions. Contrary to several of these workers, we were unable to find activator in the albumin fraction following $(NH_4)_2SO_4$ precipitation, but in the globulin fractions it occurred in the same fraction as the prothrombin. The chief point of difference between activator and prothrombin in our work (cf. Tager & Hales, 1948*a*) was the complete loss of prothrombin in plasma on repeated Seitz filtration, without any apparent loss of activator. It is conceivable that repeated Seitz filtration, while not removing prothrombin, may so alter it that although it is no longer capable of being converted into thrombin, it may yet function as coagulase activator. Since acid precipitation of this apparently prothrombin-free plasma can restore a fraction of its former activity, we cannot assume that prothrombin is ever completely absent following repeated Seitz filtration, and a physical change in its structure is therefore a possibility. Should prothrombin and activator be identical, it is not remarkable that the prothrombin of one species should be susceptible and the prothrombin of another refractory to the action of staphylococcal coagulase, since species specificity is a common occurrence in the case of most known toxins.

Both Smith & Hale (1944) and Miale (1949) produced evidence that the incubation of coagulase and activator leads to the production of a thrombin-like product, as shown mainly by a progressive shortening in clotting time of the mixture when added to guinea-pig plasma or bovine fibrinogen. Our results show such a shortening at the higher coagulase concentration tested, and the increase noted at the lower level may be due to the instability of low concentrations of coagulase when held for any period at 37°. Further evidence (Miale, 1949), that the thrombin-like product is more unstable when heated at 60° for 30 min. than coagulase or coagulase activator alone, is not borne out by our work, since we find that activator is rapidly changed at this temperature, and one may therefore expect a similar thermolability of any thrombin-like product formed by a union of the two.

It is evident from our results and those of Tager & Hales (1948*c*), that while antibodies may be produced in rabbits by successive injections of coagulase, the dose required is high and needs to be supplemented with α-lysin or to be adsorbed on a suitable carrier. Failure to use large doses, together with an adjuvant such as aluminium phosphate, probably accounts for the failure of other investigators to produce antibodies in rabbits. Our own experience indicates that an immunity comparable to that found in the sera of humans suffering from staphylococcal infections may be reached in rabbits after three intramuscular injections.

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The Ecology of the Ciliate Fauna of Hobson's Brook, a Cambridgeshire Chalk Stream

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SUMMARY: An investigation has been carried out into the association between ciliate Protozoa and bacteria in a chalk stream. The ciliates are discussed in the present paper. In general, the largest number of ciliate species and individual species were associated with a growth of green or blue-green filamentous algae. A zonation in the distribution of species along the length of the stream was observed, and particular species were found to undergo cyclical variations in the numbers of individuals. Though there was no correlation between total bacterial numbers and total numbers of ciliates present at any time, the highest bacterial numbers occurred when diatom-eating ciliates were dominant. Statistical analysis showed that the presence of Gram-negative rods in the bacterial flora was correlated with the presence of bacteria-eating ciliates. Gram-positive rods, on the other hand, were correlated with the presence of diatom-eating ciliates. The bacterial flora of the stream was in part determined from that of the surrounding soil. The periods of abundance of ciliates seemed to be related to weather conditions, since ciliates were found to increase in numbers after heavy rain or during prolonged droughts when the banks tended to crumble. Various observations suggest that the ciliate faunas of soil and brook are largely identical. The addition of a small quantity of a broth culture of a micrococcus isolated from the brook to cultures of soil ciliates in soil-extract hay-infusion medium led to a rapid multiplication of ciliates.

An investigation of the ecology of the ciliate fauna of a chalk stream (Hobson's Brook) was carried out over a period of 2 years, the immediate object being to ascertain whether Hassall (1855) was correct in his assumption that fresh-water ciliates are constantly associated with sewage pollution. This paper discusses the results obtained so far as they concern the ciliate fauna. The stream has been described in detail in a previous paper (Gray, 1951). It flows from chalk springs known as the Nine Wells, the term Nine denoting an indefinite number (Conybeare, 1897), and its fall is gradual except in a gravel reach 300 yards below the springs where the water crosses the 45 ft. contour. Nevertheless, the force of water is always considerable. Most of the springs are rheocrene in character with neutral or slightly acid waters (pH 6·6–7·0) and like other chalk springs flow at a practically constant temperature throughout the year. The biota have been described in a previous paper (Gray, 1951).

Methods

Samples were taken from the current core at twelve fixed stations along the stream in four arbitrary zones of the upper reach of the Brook (Gray, 1951); from communities of filamentous algae (*Ulothrix*, *Spirogyra*, *Cladophora*) which occurred at the sides or on the bottom of the stream, though not generally in mid-stream owing to the scouring action of the current; from the flowering plants in the centre of the Brook; from the bottom mud. When the region of maximum abundance had been determined, samples were taken only from the current core.

Samples were taken in the manner previously described (Gray, 1951). The temperature of the stream was determined at the time and place of sampling, and pH values were determined colorimetrically on return to the laboratory.

Water samples were either centrifuged or filtered through gauze and sand, and the deposit or residue examined. When filamentous algae or other macroscopic plants were present, these were agitated in the original water sample after it had been poured into a dish. The plants were then discarded and the contents allowed to settle in a conical funnel. Not less than six uniform drops of the sediment obtained by these methods was examined, and the ciliates were recorded as absent, present, or numerous according to whether 0–5, 5–10, or more than 10 individuals were present (cf. Hausman, 1917).

The numbers of individuals and of species of ciliates were generally small (Table 1). Sometimes a few individuals of several species were found, when all were recorded as present. Occasionally the drops contained large numbers of one or two species only, in which case the species present in greatest numbers was recorded as dominant and the less numerous form as subdominant. It was not uncommon to find that on a given day, six or more drops contained only a very few individuals of several species of ciliate, whereas a day or two later a sample from the same place might teem with individuals of one or two species to the exclusion of all others.

During the summer of 1947 a series of 100 bacteriological counts at 22° were made, and the total numbers and the food habits of the associated ciliates recorded. In this series of examinations a count was made of all ciliates in not less than six drops, both as regards total numbers and as regards the numbers of bacteria-eating or diatom-eating forms. At all other times only the relative abundance of ciliates was recorded (i.e. absent, present, numerous) as for example during the summer of 1948, when the morphology of the bacteria was considered in relation to the dominance of bacteria-eating or alga-eating ciliates, and the relative abundance of the dominant forms determined.

The spring waters themselves were not found to contain any ciliates, but these occurred in small pools formed in fissures of the chalk where dead leaves collected. Samples were taken from the springs throughout the investigation, but the ciliate population was negligible.

A considerable number of samplings were made at various points on cross-sections of the stream between Zone 2 and Zone 4 (Gray, 1951; Fig. 1), and it was found that on an average the greatest number and variety of ciliates occurred in the current core. To obtain a picture of the microfauna and bacterial flora in the current core, twelve fixed stations were established in relation to objects on the banks (Gray, 1951; Fig. 2). When an organism of particular interest appeared at a given point, the microfauna in this region was examined at least three times within as many days. As a routine, the microfauna at the twelve stations was examined at least once a week, samples usually being taken between 9 and 10 a.m.; occasional samples were also taken late in the afternoon and at midnight. Sampling was done working upstream.

Table 1. *Ciliates isolated from the current core of Hobson's Brook from October* 1946 *to June* 1949

The numbers indicate the Zones where the organisms were found.

HOLOTRICHA

Sub-order Gymnostomata	
Amphileptus gutta	4
Chaenia teres	3, 4
Chilodon cucullus	3, 4
Didinium nasutum	3
Holophyra sp.	2, 3, 4
Lacrymaria olor	4
Lionotopsis anser	3
Loxophyllum rostratum	2, 3, 4
Loxodes rostrum	2, 3, 4
Nassula ornata	2, 3, 4
Prorodon ovum	3
Spathidium spathula	3, 4

Sub-order Trichostomata	
Colpoda cucullus	2, 3, 4
Colpidium colpoda	3, 4
Dallasia sp.	2, 3, 4
Paramoecium caudatum	3
Paramoecidae sp.	3, 4
Pleuronema chrysalis	4
Saprophilus sp.	3, 4
Trichoda pura	2, 3, 4

HETEROTRICHA	
Condylostoma (?) sp.	3, 4
Halteria grandinella	2, 3, 4
Stentor polyphemus	3, 4
S. niger	3
Spirostomum ambiguum	3

HYPOTRICHA	
Aspidisca costata	2, 3, 4
Euplotes patella	1, 2, 3
Oxytricha gibba	2, 3, 4
Stylonichia mytilus	3, 4
Urostyla sp.	3, 4
Unidentified sp.	2, 3, 4

PERITRICHA	
Vorticella nebulifera	1, 2, 3, 4
Vorticella microstoma	3
Epistylis flavicans	3

The peritrichous ciliate *Epistylis* was only encountered once, in Zone 3; *Vorticella microstoma* was only occasionally encountered in Zone 3, but was more common in the soil on the banks of Zone 3. *V. nebulifera* was quite common in all Zones and was also found in colonies of blue-green algae floating down the stream.

Unidentified hypotrichous ciliates were common in all Zones all the year round. Some of them were morphologically identical with unidentified hypotrichous ciliates found in the soil of the surrounding land. *Stylonichia putrina* was very common in the summer of 1947 in the soil surrounding the Brook. It was not, however, encountered in the stream, where *S. mytilus* colonized Zone 3 all the year round, but was found once in Zone 2 and only on three occasions in Zone 4.

Holotrichous ciliates resembling *Paramoecium caudatum* (i.e. species of Paramoecidae) were most common in Zone 3 during the summer of 1948.

RESULTS

Association of plants and ciliates

Colonization of the Brook by the filamentous diatom *Melosira varians* was accompanied in spring 1947 and spring 1948 by an abundance of diatom-eating ciliates. In 1947 the dominant form was a *Condylostoma* species (Fig. 1), while in 1948 species of *Loxodes*, *Loxophyllum* and *Stylonichia* were abundant.

Blooms of blue-green algae found floating down the current core, from the upper third of Zone 2 to the lower third of Zone 4 in October 1946, March 1947 and January 1948, were associated with a diverse and numerous microfauna: *Halteria grandinella*, *Actinophrys sol*, *Vorticellae* sp.; Paramoecidae sp., *Pleuronema chrysalis*, *Loxodes rostrum*, *Holophyra* sp., *Colpoda* sp.; together with *Amoeba proteus* and colourless and green flagellates. Generally, the greatest variety of species of ciliates, and very often the greatest numbers, were associated with green or blue-green algal communities (cf. Picken, 1937). The age of the growth of green algae appeared to be of some importance in relation to the abundance of ciliates. Samples taken from young actively growing masses of *Spirogyra*, *Vaucheria*, *Ulothrix* and *Cladophora* had high bacterial counts, but in general very few ciliates. Older growths of green algae had high bacterial counts and a numerous and diverse ciliate fauna. Throughout the investigation the pH value of the water entangled in young blooms of filamentous green algae was often at or just below neutral point (pH 6·6–7·0), while that of mature algae was alkaline (pH 7·4). Decaying green algae and decaying leaves of green plants (with the notable exception of *Sparganium erectum*; see later) had high bacterial counts but in general very few ciliates. In contrast to what was observed in growths of green algae, the age of blooms of blue-green algae did not appear to affect the variety or numbers of associated ciliates.

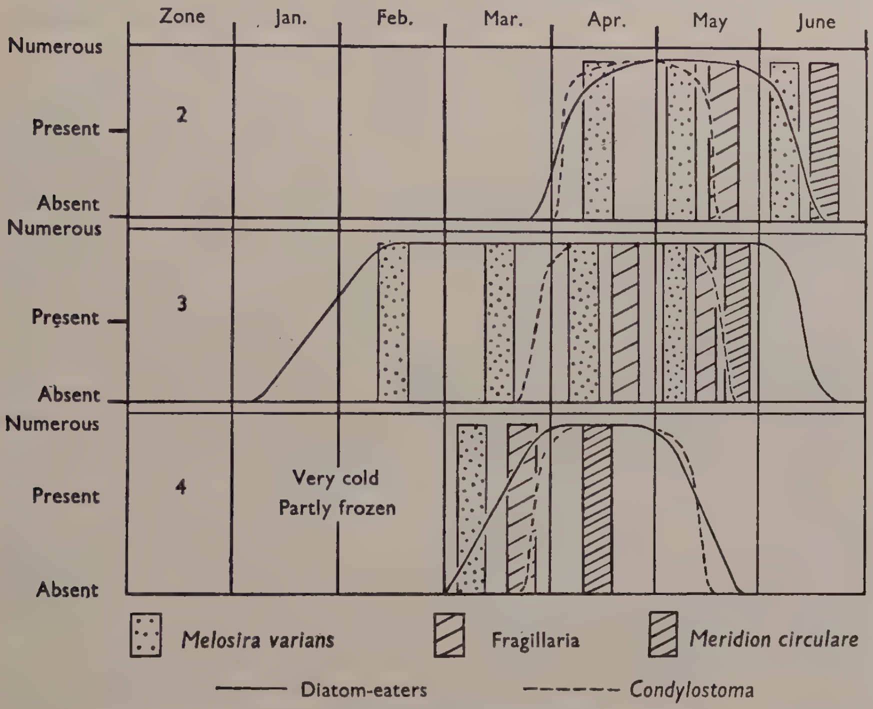

Fig. 1. The abundance of diatoms and of diatom-eating ciliates in the stream during January to June 1947.

On the four occasions when blooms of *Oscillatoria* occurred the pH value of the water was *c.* 7·4 and did not vary appreciably with the age of the bloom.

The leaves of green flowering plants in general had a poor ciliate population, but the leaves of *Sparganium erectum* provided a marked exception. Perhaps because of their virtual confinement to the centre of the stream, and their vertical growth (which may prevent the accumulation of silt on the leaf surface) they were usually coated with algae and diatoms and at all ages bore a numerous and varied ciliate fauna.

Zonation of ciliate species

Only a few species of ciliates were recorded. The total numbers of any one species varied within wide limits but during the investigation the same species constantly recurred. The respective Zones each tended to have their characteristic microfauna, as if the topography and physical factors defined a distinct environment in each Zone. Thus when diatom-eating ciliates were dominant, *Loxodes rostrum* and *Loxophyllum rostratum* were found in Zone 2, *Stylonichia mytilus* in Zone 3, and only *Nassula ornata, Euplotes patella* and certain Oxytrichidae in Zone 4. Throughout the investigation such zonation was repeatedly encountered. The most striking example was provided by *Stylonichia mytilus*. This organism was virtually confined to Zone 3. It was only found once in Zone 2 and once or twice in Zone 4, and was usually the most common ciliate in Zone 3. Although this zonation was marked with the diatom-eaters, it also occurred, though to a lesser degree, among the bacteria-eating forms. A large diatom-eating form (a *Condylostoma*) dominated the whole rural reach in March 1947 to the virtual exclusion of all other ciliates, but disappeared 6 weeks later. It was not observed again until the autumn of 1948, when it was found at the junction of Zones 2 and 3, the point at which it was first recorded in January 1947.

Cycles of abundance of ciliates

The abundance of individual members of the ciliate fauna varied considerably at different times, and a sequence of species and series of maximum and minimum phases of individual abundance was observed. One ciliate tended to succeed another; bacteria-eaters might precede or succeed diatom-eaters. Whatever form was first observed to be dominant, it eventually ceased to be dominant or disappeared completely in periods from a few hours to 4 days, to be succeeded by another form not necessarily of related food habits. Once the new form had established itself, it might be dominant or share dominance with the preceding form, or both might disappear and be replaced by a third form (Fig. 2).

During a dominant phase, the given ciliate spread upstream and downstream, and in the minimum phase retreated to the upper levels. Thus in May 1947 a bacteria-eating ciliate, a *Colpoda* sp., spread from Zone 2 to the upper third of Zone 4. By the end of June it had retreated to the upper third of Zone 2 and it was not again recorded from Zone 3 until the middle of July. A species of

Holophyra present in Zone 3 in June 1947 was found throughout Zone 2 to Zone 3 and Zone 4 by the end of the month; subsequently it was only found in the upper third of Zone 2. *Halteria grandinella* spread in similar manner between Zone 2, Zone 3 and Zone 4. During the summer of 1947 *Stylonichia mytilus* colonized Zone 3; it was not found in Zone 2 and only on one or two occasions in Zone 4. In its maximum phase it was dominant in Zone 3 and occasionally spread to the upper third of Zone 4; during its minimum phase it retreated to the upper third of Zone 3. *Paramoecium caudatum*, a bacteria-eater, was only occasionally observed in 1947 but from Zone 3 it spread into Zone 4, retreated to Zone 2 and later spread through Zone 3 into Zone 4 again.

In 1947 and 1948 it was observed on several occasions that during the maximum phases of many of the ciliates (e.g. *Stylonichia mytilus* and other Hypotrichidae, *Loxodes rostrum*, *Chilodon cucullus* and species of *Colpoda* and *Holophyra*) forms in binary fission and conjugation were present. *S. mytilus*, for example, was observed in conjugation once, and in fission four times, in June 1947 during the weekly samplings at the twelve fixed stations, and in conjugation once, and in fission three times, in July 1948 during the twice a week samplings carried out at stations 6–8. In 1947 this reproductive phase was often followed by the appearance of small forms and a diminution in numbers; this was observed on twelve occasions in June in the weekly samplings from stations 1–12. In 1948, however, the maximum phase was often succeeded not only by the appearance of small forms of all species of ciliates, on twenty occasions in July in twice-weekly samplings at stations 6–8, but also by the appearance of monstrous forms, double monsters, and bent and swollen forms with irregular outlines. The latter were noted on sixteen occasions in July 1948 in the twice-weekly samplings from stations 6–8, when abnormal forms of *Paramoecia* were observed on six occasions and abnormal Hypotrichidae on five occasions. These abnormal forms tended to occur in the lower reaches, they were almost wholly limited to station 8 of Zone 3. It was often found that active healthy forms of a particular species were abundant at station 6, present in diminished numbers at station 7, and grossly distorted at station 8.

On the whole, the bacteria-eating *Paramoecium caudatum* and other *Paramoecia* were more common, especially in Zone 3, during 1948, than in 1947. They were never observed in conjugation but seen once in fission in July 1948 during an exceptionally abundant maximum phase.

Abundance in relation to numbers of bacteria

Although it is convenient to divide the ciliates into two groups according to their feeding habits (diatom-eaters and bacteria-eaters) the division is not absolute. Some diatom-eaters, e.g. *Stylonichia mytilus*, will ingest bacteria in the absence of diatoms, as Sandon (1932) found, or as epiphytic bacteria on diatoms. Harvey (1945) found the greatest numbers of bacteria in sea water in the samples richest in phytoplankton. It seems likely therefore that diatom-eating ciliates will also tend to diminish the numbers of bacteria present, either because they are ingested accidentally as epiphytes on diatoms, or because they are an alternative food for certain species. A study of the relationship between the numbers of individuals and of genera of ciliates, and the number and kinds of bacteria present in the Brook, was made during the summers of 1947 and 1948. A series of 100 bacteriological counts of total numbers of bacteria at 22° were made in parallel with collections of ciliates at the twelve stations during June-July 1947. When the figures obtained were analysed statistically, no correlation was found between total bacterial numbers and total numbers of ciliates present at any time; nor were fluctuations in total bacterial numbers correlated with fluctuations in total ciliate numbers. It was found, however, that the highest bacterial numbers (up to 400,000/ml.) occurred when diatom-eaters were dominant.

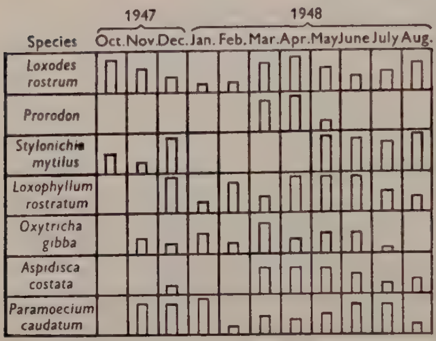

Fig. 2

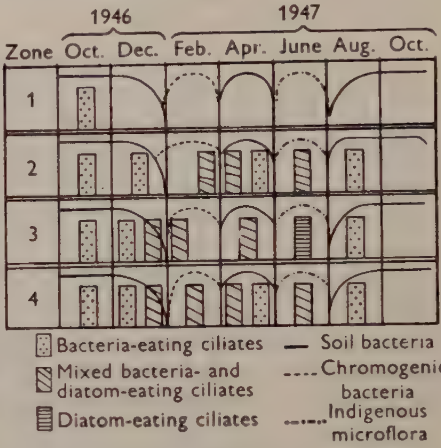

Fig. 3

Fig. 2. The relative abundance of seven ciliates in Zone 3 (the middle third of the Brook) from October 1947 to August 1948.

Fig. 3. The prevailing microflora as shown by bimonthly examinations and the dominant ciliate fauna from October 1946 to October 1947.

A series of fifty samples taken in May–June 1948 from stations 6–8 in Zone 3 showed no correlation between the total ciliate numbers and the total bacterial numbers at 22° but, as in 1947, the highest bacterial numbers were associated with a dominance of diatom-eaters. When the number of bacterial colonies was small (six to twelve) these were picked off and stained by Gram's method. With counts of 50–100, twenty colonies were selected at random, picked off and stained. This examination of the bacteria suggested that dominance of diatom-eaters or of bacteria-eaters was associated with the presence or absence of certain types of bacteria. There seemed to be some correlation between the presence of diatom-eaters and Gram-positive rods, while bacteria-eaters were associated with Gram-negative rods, and abundance of both groups was associated with micrococci. Thus from fifty samples taken at five stations (ten samples per station) on twelve occasions bacteria-eating ciliates were dominant when Gram-negative rods were more abundant than Gram-positive rods, or when only Gram-negative rods and micrococci were present. Diatom-eating ciliates were dominant on four occasions when Gram-positive rods were more abundant than Gram-negative, and on two occasions when Gram-positive rods and micrococci were equally abundant. Statistical analysis of the data by Mr D. V. Lindley (Mathematical Laboratory, Cambridge) showed that the correlation of Gram-negative rods with the presence of bacteria-eaters and of Gram-positive rods with diatom-eaters was statistically significant.

These findings were supported by other observations made in the field. When either Gram-positive or Gram-negative bacteria dominated the microflora of the stream, one or other of the two groups of ciliates tended to be dominant (Fig. 3). When the microflora was largely composed of Gram-negative rods, bacteria-eaters (e.g. *Colpoda* spp.) were common; when Gram-positive rods were more numerous than Gram-negative rods, diatom-eaters supplanted bacteria-eating forms. The bacterial flora of the stream came largely from the surrounding land, as shown by the appearance of soil bacteria after heavy rain or periods of drought (Gray, 1948, 1951) and the character of the soil bacteria tended to be modified by agricultural operations. In a tributary stream flowing into the Brook from a farmyard and Shelford village (Gray, 1951) the greatest numbers of *Paramoecia* were found where sewage pollution (i.e. by Gram-negative rods) was highest. Where the tributary entered the Brook and sewage pollution was least, *Paramoecia* disappeared and diatom-eating ciliates became prominent (Gray, 1951).

The influence of weather conditions

The cycles of abundance of ciliates could not be related to total numbers of bacteria growing at 22°, pH value or temperature of the water. *Stylonichia mytilus*, however, appeared to exhibit lunar periodicity (see later). With this exception, although the cycles could not be related to any one of the above factors, they were related to weather conditions. The ciliates increased in numbers and spread down the stream after floods or heavy rain, or during prolonged periods of hot dry weather when the banks tended to crumble and soil fell into the water (Fig. 4). The latter occurred particularly in 1947, when floods in the spring were succeeded by months of drought. By contrast, the summer of 1948 was dull and wet. In this year, in twenty-two examinations over the entire length of the reach of the Brook examined, thirteen were made within 24 hr. of storms or heavy rain. In the samples taken during eight of these latter examinations, bacteria-eating ciliates were dominant; on three occasions diatom-eaters were dominant, while on two occasions dominance was shared between bacteria-eating and diatom-eating ciliate protozoa. It did not seem probable that this phenomenon was due solely to mechanical transport by the increased volume of water, for the maximum peak of abundance of a particular species had often passed at a particular point while a spate was still passing downstream or while rain was still falling. It was noted that the actual numbers of individuals of particular species (rather than of all species) increased at the sampling points as they did when soil was crumbling into the water; that is to say, only one species out of perhaps half a dozen originally present would increase in number at a particular sampling station.

Lunar periodicity in Stylonichia mytilus

When the numbers of this organism were plotted against the date, it was found that during 1947 maximum phases occurred on an average 7–10 days after a new moon or a full moon, especially the latter. The two largest maxima, when the organism dominated the whole of the middle reach of the stretch under examination, occurred 9 days after a new moon in July and 4 days after a full moon in December. By contrast with the dry weather and clear nights of 1947, the year 1948 was wet and dull, and *S. mytilus* was only recorded from May to September. Nevertheless, its numbers rose to a maximum on five occasions averaging 8 days after a new or a full moon. A statistical analysis of the numbers of *Stylonichia* (as obtained by the methods described) considered in relation to lunar periodicity in days, was made by Mr D. V. Lindley, and suggested that *S. mytilus* has a maximum about 4–7 days before and after a full moon, with some slight evidence for a fall in numbers at the full moon. More data would be necessary to establish the possible periodicity (Gray 1951 *a*).

Possible influence of the macrofauna

One of the features of the changing fauna of the Brook was a peak of numbers of dipterous insects in late summer (Gray, 1951). Although the relative abundance of individuals of various species of ciliate continued all the year round, their total numbers fell steadily throughout the summer. During this period there was an increase of dipterous larvae, especially chironomids and simuliids; these were particularly common in Zone 3. They are known to be omnivorous microphages. Since they occur in maximal numbers in the current core, where the largest ciliate population was found, it is possible that the fall in total numbers of ciliates from the current core during the summer is correlated with this enormous increase in the population of microphagous larvae (Fig. 5). Barker (1946) found a similar association in sewage filter beds, where seasonal peaks of fly output were accompanied by a depression of ciliate protozoa.

Identity of soil and stream ciliates

The numbers of ciliates at the sampling points increased greatly after heavy rain or during periods of drought. Especially after rain, the increase in numbers was too great, too sudden, and too general, wholly to be accounted for by multiplication of the existing ciliate population as a result of some change in natural conditions. Moreover, soil bacteria appeared in the microflora at such times (Gray, 1948). It was considered possible, therefore, that not only soil bacteria but also soil ciliates were entering the Brook. When the soil ciliate population of the banks of the stream and of the soil 200 yards away on the banks of ditches emptying into the Brook was examined, the same genera were found to be present as in the stream (i.e. *Colpoda*, Oxytrichidae, *Halteria*). Ciliates of the same genus as those in the stream were also obtained from the interior of clods of dried mud originally excavated from the stream-bed and cast on adjoining fields. These observations (Table 2) suggested that the ciliate faunas of the soil and Brook are virtually identical, one habitat receiving recruits from the other (Gray, 1948).

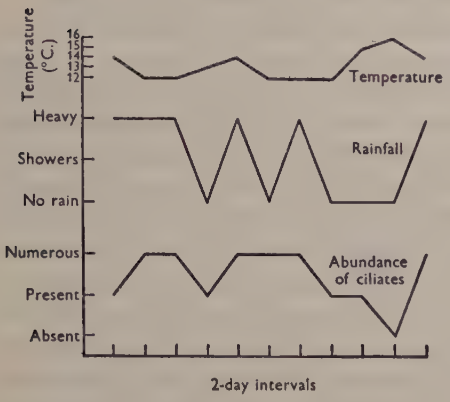

Fig. 4

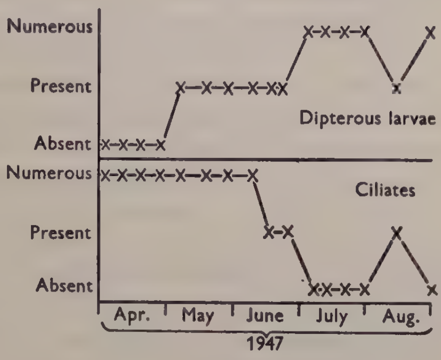

Fig. 5

Fig. 4. Rainfall, temperature, and abundance of ciliates at 2-day intervals in Zone 3 (the middle third of the Brook) during June and July 1948.

Fig. 5. The abundance of dipterous larvae and of ciliates in Zone 3 (the middle third of the Brook) during the summer of 1947.

Table 2. *A comparison of the infusoria obtained from the soil round Hobson's Brook in the early summer of* 1947 *as compared with forms found in the Brook during the investigation*

In the soil	In Hobson's brook
Ciliata	
Colpidium colpoda	*Colpidium colpoda*
Colpoda cucullus	*Colpoda cucullus*
Halteria grandinella	*Halteria grandinella*
Holophyra sp.	*Holophyra* sp.
Holotrichous sp.	*Holotrichous* sp.
Paramoecidae	Paramoecidae
Oxytricha sp.	*Oxytricha* sp.
Oxytrichidae	Oxytrichidae
Spirostomum ambiguum	*Spirostomum ambiguum*
Vorticella microstoma	*Vorticella microstoma*
Rhizopoda	
Actinophrys sol	*Actinophrys sol*
Amoeba limax	*Amoeba limax*
A. proteus	*A. proteus*
Testaceous rhizopods	
Difflugia sp.	*Difflugia* sp.
Arcella	Not found
Rotifera	
R. vulgaris	*R. vulgaris*
R. elongatus	Not found; but found in River Cam
Philodina sp.	*Philodina* sp.

Multiplication of soil ciliates in the presence of broth cultures of micrococci and pseudomonads

Cultures of soil ciliates were obtained by covering a few grams of soil in a conical flask with sterile tap water containing 0·2–0·5 % (v/v) hay infusion and 1 % (v/v) soil extract. The hay infusion was prepared from good quality hay as tea is prepared (Heath, n.d.), the soil extract was prepared according to the formula of Cunningham (1947). The numbers of ciliates in cultures made with this medium increased but slowly when the sole food supply was the natural soil microflora. Ciliates were not found in the cultures until these had been incubated at room temperature for several days or even as long as 2–3 weeks. Following the observation of Singh (1946) cultures were inoculated with micrococci from the stream. A massive multiplication of ciliates resulted in cultures so inoculated. A few drops of broth cultures, or of filtrates of broth cultures, of two micrococci 3/2 and 3/3 isolated from Zone 3 in the summer of 1947, caused a great multiplication of the soil bacteria, initially of spore-forming bacteria, followed in a few hours by a great increase in the numbers of soil ciliates. Strain 3/2 had not so powerful an action as 3/3 and died out within a short time, whereas 3/3 was subcultured for 3 years and provoked an identical action at the end of that time. It retained its morphology, showing that it was not a pleomorphic strain of *Bact. globiforme* Conn. A few drops of broth only caused a multiplication of bacteria in cultures of soil ciliates but no increase in the numbers of the ciliates. During the increase in numbers of ciliates after inoculation with broth cultures of micrococcus 3/2 or 3/3, ciliates were seen in fission. A few drops of hay-infusion cultures of micrococcus 3/2 or 3/3, or filtrates of such cultures, did not cause either a multiplication of bacteria or an increase in the numbers of ciliates. There was, however, some increase in the numbers of flagellate protozoa. After a few drops only of broth cultures of micrococcus 3/2 or 3/3 had been added, the bacterial flora of the culture at the time the numbers of soil ciliates were increasing, was composed largely of long and short Gram-negative rods, among which pseudomonads were most common, together with the strain of micrococcus that had been added, some chromogenic micrococci, and an occasional spore-former. Four to seven days after multiplication of the ciliates had ceased, the microflora was almost wholly composed of long and short Gram-negative rods (pseudomonads prominent) and the micrococcus.

These experiments showed that increase in the numbers of bacteria-eating soil ciliates followed inoculation of hay-infusion soil-extract cultures with a few drops of the micrococcus culture; no experiments were undertaken to establish whether micrococci alone among the stream microflora were capable of stimulating this increase.

Single cysts of *Colpoda* and *Colpidium* spp. were picked out from mixed cultures of soil ciliates, and pure cultures of these bacteria-eating ciliates were obtained by passing them through a series of sterile baths. It proved possible to remove gross bacterial contamination by such methods, but the cysts could not be freed from a Gram-negative rod. This was a species of *Pseudomonas* identical with that isolated from mixed cultures of soil ciliates after addition of micrococcus 3/2 or 3/3.

Cultures of *Colpoda* could be established successfully in liquid media containing no bacteria except the pseudomonas, and in media containing only the pseudomonas and a brook micrococcus, by transferring single individuals or cysts to the media in question. It was observed, however, that although the ciliates multiplied in the presence of such bacterial foods, the multiplication was not unlimited. Pure cultures of *Colpoda* and *Colpidium* grew and multiplied during the first 24–48 hr. but multiplication then ceased and some ciliates encysted, although food bacteria were still abundant in the medium and would support vigorous multiplication of soil ciliates if reinoculated.

DISCUSSION

The conclusion reached by Hassall (1850, 1855), that ciliates (notably Paramoecidae) are constantly associated with sewage pollution because they find in pollution 'their means of subsistence and growth', probably marks the first tacit recognition of the dependence of certain ciliates on a supply of bacteria rather than green algae as a source of food. Two years later, in 1852, Pritchard published the third edition of his *Infusorial Animalcules* in which the nutrition of ciliates is only vaguely indicated: 'The digestive cells of Paramoecidae are demonstrated in one species by its usual green food....In all Kolpoda digestive cells have been indicated by coloured foods....' Hassall's observations were largely made on the Thames; but in spite of the importance of sewage pollution of rivers, they are only paralleled in modern times by those of Lackey (1938) on ciliates as pollution indicators in the Scioto River.

Cycles of maximum and minimum abundance of different species of ciliates, noted in the Brook, have been recorded by many workers (Calkins & Summers, 1941); they also occur in the soil (Russell, 1923). The present study suggests that in many instances they are directly related to climatic conditions, since peaks of abundance occur after heavy rain, or after drought when soil is washed or crumbles into the Brook. At such times there was a peak of abundance of soil bacteria in the Brook and a maximum of bacteria-eating ciliates.

It was noted, however, that the peak of ciliate numbers (especially bacteria-eaters) which followed pollution of the Brook by soil formed so rapidly that it could hardly be accounted for by simple multiplication of the existing population induced by an increased food supply. Since ciliates occur in the soil, it was thought that they might have entered the stream with soil bacteria. The suggestion was therefore made that soil and fresh-water ciliates are in fact identical, one habitat being the source of the other's population (Gray, 1948). Soil (ciliate) protozoa were known to Ehrenberg (1838), and many later workers have commented on their similarity to fresh-water forms. Russell (1923) observed that at one time it was held that the soil was recruited at random by protozoan cysts carried by wind from dried-up ponds and streams, but added that to 'Martin & Lewin (1915) must be ascribed the distinction of first proving that the soil possesses an active population'. Again, it was suggested by Calkins & Summers (1941) that 'water-dwelling forms might occasionally be found in soil and interpreted as casual soil-dwelling types'. Woodruff (1912), on the other hand, in a classical study of the ciliates of hay-infusions, gave air, water, and hay as their source, but did not mention soil; similar sources, exclusive of soil, were given by Kent (1881). An examination of the microfauna of the soil round the Brook revealed that, with the exception of species of *Stentor*, all the ciliates isolated from the Brook during the 2½ years of this inquiry were forms recorded by Sandon (1927) as common in the soil, to an extent suggesting that the soil may be the chief source of the ciliate population of inland waters.

Since it is known that individual crop-plants are associated with characteristic bacterial flora, it was of interest to examine the nature of the crops surrounding the Brook during the investigation. During this period, the crops sown on adjoining land which largely drained into the Brook, were almost wholly a cereal rotation (Gray, 1951). Wheat and barley were sown on each bank of Zone 3 during 1947; linseed and wheat, during 1948. The banks of Zone 4 were sown with oats and sugar-beet, and rape-cole and kale, in 1947; with oats and wheat, in 1948. One bank of Zone 2 is old permanent grassland; while the other bank was sown with barley in 1947, and with meadow hay in 1948. Lochhead, Timonin & West (1940) showed that the rhizospheres of plants, notably cereals, roots, and clover, are characterized by a distinct microflora, among which Gram-negative rods are very abundant. It seemed possible, therefore, that the crop-rotation on the banks was providing bacteria-eating ciliates with their chosen bacteria, viz. Gram-negative rods, since bacteria-eating ciliates were dominant when soil entered the Brook.

Gram-negative rods derived from the rhizosphere would be most plentiful when the crops were growing, that is, in the late spring and early summer. When the land was ploughed and dressed with farmyard manure other Gram-negative rods (e.g. *coliform* bacteria) would enter the stream, while many would be derived from the droppings of sheep then folded beside the Brook. Severin (1895; quoted by Waksman, 1927) found that bacterial rods predominated in freshly ploughed soil, but that within 2 weeks micrococci were abundant. I found that micrococci tended to dominate the microflora of the Brook in the autumn (Gray, 1951).

Singh (1941) showed that the bacterial food supply is the chief factor controlling the abundance of soil Protozoa. The restricted number of species of soil ciliates found during this investigation may be attributable to the same cause; for if soil and stream ciliates are identical, the same factor of bacterial food supply would tend to restrict the number of species found in the Brook.

Anscombe & Singh (1948), in an investigation on the limitation of bacteria by micro-predators in soil, showed that two species of Myxamoebae (*Dictyostelium giganteum*, *D. mucoroides*) and two species of Myxococcaceae (*Myxococcus fulvus*, *Chondrococcus exiguus*) prefer Gram-negative bacteria. The cycles of abundance of ciliates may therefore be attributed to variations in the bacterial food supply and to variations in the numbers of soil ciliates entering the Brook. Bacteria-eating ciliates seem to be particularly favoured at those times when Gram-negative rods are abundant in the soil microflora. There appears to be a division among the bacteria-eaters themselves according to the character of the Gram-negative rods, for Paramoecidae were only abundant when *Bact. coli* was numerous, as in the tributary, whereas other forms (e.g. *Colpoda*) were associated with abundance of Gram-negative rods not strains of *Bact. coli*. In this respect the investigation supports the emphasis laid by Noland (1925) on the importance of the food supply in the ecology of fresh-water ciliates.

In connexion with the occasional presence of ciliate monsters in the Brook, and the apparent importance of a micrococcus in determining the abundance of the Brook ciliates it is interesting that Sonneborn (1932) induced the formation of double-monsters and chain-forms in *Colpidium campylum* by feeding this ciliate on a strain of *Micrococcus sensibilis*. The two strains, 3/2 and 3/3, of micrococcus isolated in the present work were not identified. It was noted that double-monsters and other abnormal forms of both diatom-eating and bacteria-eating ciliates (e.g. *Loxodes rostrum* and Paramoecidae) were present on more than one occasion when unidentified micrococci dominated the bacterial flora of the brook. They were particularly noticeable among the Paramoecidae, though in addition abnormal forms of *Loxodes*, *Loxophyllum rostratum* and Oxytrichidae were also noted. Certain of them closely resembled Sonneborn's figures of degenerate *Colpidium campylum* fed on a strain of *Micrococcus sensibilis*.

It is suggested, therefore, that the periods of ciliate abundance may be induced by the nature and quantity of the bacteria made available as food by climatic conditions, when these cause a massive and general contamination of the stream with soil bacteria and ciliates.

Noland (1925) emphasized the supreme importance of food supply in the ecology of fresh-water ciliates; the present inquiry, in stressing this conclusion, points to the soil surrounding the aqueous habitat of the ciliates as the major source of food. Diatoms also may perhaps come from the soil, but both diatom-eaters and bacteria-eaters ingest bacteria which may also be derived from the soil. Climatic conditions may be responsible for the quantity, and present and past agricultural operations for the quality, of the food bacteria provided. The quantity of suitable bacteria available in the soil at any one time will vary, for Taylor (1936) has recorded short-period fluctuations in the numbers of bacterial cells in soil. While fresh-water ciliates may justly be regarded as indicators of bacterial pollution, the present study has shown that pollution may derive from the soil in the absence of sewage as such. The ecology of the fresh-water ciliates suggests how close is the association between the stream and the soil through which it runs.

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The Mechanism of Propionic Acid Formation by *Clostridium propionicum*

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SUMMARY: *Clostridium propionicum* evidently possesses a mechanism for the conversion of lactate to propionate different from that found in the previously studied propionic acid producing bacteria. Reactions common to certain propionibacteria and *Veillonella gazogenes* which could not be demonstrated with *Cl. propionicum* include: (*a*) the decarboxylation of succinic acid; (*b*) the fermentation of malate and fumarate; (*c*) the variation in the ratio of acetic to propionic acid according to the concentration of CO_2; (*d*) the fixation of CO_2 in propionic acid.

Two mechanisms have been proposed for the formation of propionic acid from lactate by propionibacteria (Werkman & Wood, 1942): (*a*) the dehydration of lactate to acrylate which is then reduced to propionate; (*b*) the formation of succinic acid and its subsequent decarboxylation, i.e. lactate → pyruvate ⇌ oxalacetate ⇌ malate ⇌ fumarate → succinate → propionate + CO_2. The propionibacteria are unable to ferment acrylic acid, so the first mechanism is untenable for these organisms. Evidence has been presented for strains of *Propionibacterium* (Delwiche, 1948; Johns, 1951*b*) and for *Veillonella gazogenes* (Johns, 1951*a*), which indicated that (*b*) represents the probable pathway for propionate formation in these bacteria. Cardon & Barker (1947) have shown that *Cl. propionicum*, an organism isolated from marine mud, decomposes lactate, acrylate and pyruvate to give acetic and propionic acids and carbon dioxide. The present paper gives results of experiments which attempted to demonstrate with *Cl. propionicum* reactions common to propionibacteria and *V. gazogenes*.

EXPERIMENTAL

Methods

The methods were essentially the same as those used previously (Johns, 1951*a*). The Van Slyke-Folch wet oxidation as used by Barker (1949*a*) was employed instead of the Pregl micro-combustion for the conversion of propionate and acetate to carbonate.

The strain of *Cl. propionicum* used was isolated by the enrichment culture method (Cardon & Barker, 1946) from a heap of decomposing grass clippings. The organism obtained readily fermented alanine, lactate, pyruvate, serine and threonine, failed to ferment sugars, and appeared in every way identical with the strain described by Cardon & Barker (1946).

The medium used for growth and the method of preparing washed cell suspensions were those of Cardon & Barker (1947). Cultures were harvested after 16 hr. growth at 37°.

RESULTS

Succinic acid decarboxylation

Washed suspensions of *Cl. propionicum* were tested by the Warburg technique for their ability to ferment succinic acid. The Warburg cups contained 1 ml. bacterial suspension (*c.* 20 mg. dry wt./ml.), 1·3 ml. M/15 phosphate buffer, 0·2 ml. 0·2 M sodium succinate. The gas phase was nitrogen and the temperature of incubation 37°.

Over the range pH 5·3–7·6 there was no change of gas pressure in the manometers. The experiment was repeated with 0·1 ml. of 0·1 M succinate in the presence of an equimolar quantity of alanine. These combined substrates gave no greater gas production than that given by alanine alone. By the same technique it was shown that DL-malate and fumarate, at pH values between 5·0 and 7·6, were not fermented by washed suspensions of the organism grown on either alanine or lactate.

The effect of sodium fluoride on fermentation of lactate and pyruvate

Barker & Lipmann (1944), using *Propionibacterium pentosaceum,* found that in presence of sufficient fluoride entirely to prevent lactate reduction, pyruvic acid was reduced to propionic acid. The influence of sodium fluoride on the fermentation of lactate and pyruvate by washed suspensions of *Cl. propionicum* was determined by the effect on the rate of CO_2 production. The rates of both dissimilations were progressively inhibited to a similar extent by increasing concentrations of fluoride (Table 1).

Table 1. *Fluoride inhibition of lactate and pyruvate fermentations by* Clostridium propionicum

Manometer flasks contained 1·5 ml. of bacteria (28 mg./ml.) in 0·1 M phosphate buffer (pH 6·8); substrate 0·1 ml. 0·1 M sodium pyruvate or 0·2 ml. 0·1 M sodium lactate; NaF as indicated; 0·2 ml. 2N-H_2SO_4; total volume 2·5 ml. Gas phase N_2. Temp. 37°. Acid tip after 20 min.

NaF concentration (1 %)	Percentage inhibition	
	Lactate	Pyruvate
0	0	0
0·01	19	23
0·02	39	42
0·04	76	80
0·10	87	92
0·20	92	95

Since the effect of fluoride on the fermentation of lactate and pyruvate by washed suspensions of *V. gazogenes* had not previously been determined the experiment was repeated using a washed suspension of this organism (Johns, 1951*a*). The gas produced in this case was hydrogen and carbon dioxide; the results are shown in Table 2. Although the fermentation of lactate is more sensitive to fluoride than is that of pyruvate, complete inhibition was not achieved even with 0·2 M-NaF.

Table 2. *Fluoride inhibition of lactate and pyruvate fermentations by* Veillonella gazogenes

Manometer flasks contained 1·5 ml. of bacteria (27 mg./ml.) in 0·1 M phosphate buffer (pH 6·8); substrate 0·1 ml. 0·1 M sodium pyruvate or 0·2 ml. 0·1 M sodium lactate; NaF as indicated; 0·2 ml. 2N-H_2SO_4; total volume 2·5 ml. Gas phase N_2. Temp. 37°. Tip after 10 min.

NaF concentration (*M*)	Percentage inhibition	
	Lactate	Pyruvate
0	0	0
0·0004	0	—
0·002	55	—
0·004	82	—
0·01	88	25
0·02	90	38
0·1	93	44
0·2	94	45

Effect of CO_2 *concentration on fermentation of lactate*

The concentration of carbon dioxide influences the ratio of propionic to acetic acid formed by the fermentation of lactic acid by propionibacteria and by *V. gazogenes* (Johns, 1951*a*, *b*). Increased CO_2 concentration produced a greater CO_2 fixation leading to an increased amount of propionic acid. Similar experiments were carried out with *Cl. propionicum* using three tubes of the type used by Barker (1949*b*). Each contained 26·5 ml. of fluid made up of 25 ml. of Barker's medium (pH 7·18), 1 ml. sodium lactate (0·3 g./ml.) and 0·5 ml. 1 % sodium sulphide. In tube (*a*) carbon dioxide was absorbed in alkali during incubation. In tube (*b*) 1 ml. of sterile N sodium carbonate was added after autoclaving (the pH of the buffer was adjusted so that the final pH after addition was 7·17). This tube was gassed with sterile CO_2 and sealed so that pressure increased by the amount of CO_2 produced during fermentation. Tube (*c*) was similar to (*a*) except that the pH was 5·95. After inoculation the tubes were incubated for 48 hr., and the ratio of volatile acids determined chromatographically (Elsden, 1946). The ratio propionic: acetic acid was 1·96 in tube (*a*), 1·93 in tube (*b*) and 1·95 in tube (*c*), compared with a ratio of 2 as found by Barker (1949*b*). All the lactate was fermented in each case.

CO_2 fixation experiments

Wood, Werkman, Hemingway & Nier (1941) working with propionibacteria and Johns (1951*a*) with *V. gazogenes* showed that during fermentation these organisms fixed $C^{13}O_2$ in the carboxyl group of the propionic acid produced. The same technique as used for *V. gazogenes* (Johns, 1951*a*) was used to determine whether *Cl. propionicum* could utilize $C^{13}O_2$ in the formation of propionic acid.

The medium used was as in tube (*b*) above, with the sodium carbonate in the form of $Na_2C^{13}O_3$ (18·1 atoms % excess C^{13}). The complete experiment was carried out in duplicate. The mass spectrograph determinations of the C^{13}

content of products which were separated chromatographically (Elsden, 1946) showed in no instance any excess of C^{13} over that in the control sample, indicating that there had been no fixation of carbon dioxide in either acetate or propionate.

DISCUSSION

The results indicate that *Cl. propionicum* decomposes lactate to give propionic acid by a type of fermentation different from that observed with *V. gazogenes* (Johns, 1951*a*) and propionibacteria (Krebs & Eggleston, 1941; Delwiche, 1948) which ferment malate and fumarate readily and decarboxylate succinate to acid and carbon dioxide, whereas *Cl. propionicum* does not. The ratio acetic: propionic acid, as determined in fermentations by propionibacteria, varies greatly according to conditions of growth. This depends mainly on the CO_2 tension (Johns, 1951*b*), which determines the amount of succinate formed, and on the final pH value of the medium, which determines the amount of succinate decarboxylated. With *Cl. propionicum* variation in CO_2 tension and pH of the medium have no effect on the acid ratio, in agreement with the observation that succinate appears not to be an intermediate in propionate formation by this bacterium.

Barker & Lipmann (1944) found with *P. pentosaceum* that, whereas lactate decomposition was practically stopped by 0·00625 M fluoride, pyruvate decomposition was only 30 % inhibited at this concentration; even with 0·1 M fluoride, pyruvate was still fermented at an appreciable rate. They also found that, with lactate reduction blocked, little or no lactate accumulated when pyruvate was decomposed. This was interpreted to mean that lactate was probably not here the pathway of pyruvate reduction to propionate. With *V. gazogenes* lactate fermentation is much more sensitive to fluoride than pyruvate fermentation (Table 2). However, lactate reduction is not completely eliminated by a high concentration of fluoride. In contrast to the dissimilation of lactate and pyruvate by propionibacteria and by *V. gazogenes*, this dissimilation by *Cl. propionicum* is progressively inhibited by increasing fluoride concentrations to very nearly the same degree, indicating that lactate may be on the pathway of pyruvate reduction to propionate by this organism. As shown by Cardon & Barker (1947) *Cl. propionicum* ferments acrylic acid, whereas *Propionibacterium pentosaceum* does not (Barker & Lipmann, 1944). The final piece of evidence for the existence of two separate mechanisms for propionic acid formation is that whereas *Cl. propionicum* does not utilize CO_2 for propionic acid production, propionibacteria (Werkman & Wood, 1942) and *V. gazogenes* do (Johns, 1951*a*). It seems probable that the route of propionic acid formation from lactate by *Cl. propionicum* is lactate → acrylate → propionate; but there is no evidence yet for the formation of acrylate during fermentation. It seems certain from the work of Carson (1948) that there is still more to be learnt about the mechanism of propionic acid formation in bacterial fermentations. He has demonstrated a conversion of acetate to propionate which is not explained by the two schemes proposed by Werkman & Wood (1942).

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Competitive and Non-competitive Inhibitors of Bacterial Sulphate Reduction

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SUMMARY: The selenate ion, a remarkably powerful competitive antagonist of the reduction of sulphate by *Desulphovibrio desulphuricans* (Hildenborough) suspensions, did not affect reduction of sulphite and thiosulphate. Cysteine, methionine and glutathione had no anti-selenate effect. Selenate also inhibited the growth of *D. desulphuricans*; this effect was antagonized competitively by sulphate and non-competitively by sulphite. Repeated subculture in subinhibitory selenate + sulphate mixtures did not give rise to a selenate-resistant strain, though selenium was deposited in these conditions.

The monofluorophosphate ion behaved similarly: it was a competitive sulphate antagonist in growth and in sulphate reduction, though it had a lower specific anti-sulphate activity than selenate. It did not affect the metabolism of sulphite or thiosulphate. At high concentrations it showed non-competitive inhibition of sulphate reduction.

Of the other 'analogues' of sulphate tested, potassium tellurate suspensions and chromate inhibited growth and sulphate reduction, but were not competitive sulphate antagonists. High concentrations of perchlorate depressed sulphate reduction in a non-competitive manner, and methanesulphonate, β-hydroxyethanesulphonate, benzenesulphonate, ethylsulphate, sulphamate and dimethylsulphone were inactive.

An acridinium dye known to inhibit the growth of *D. desulphuricans* did not affect sulphate reduction, and, in growth, was not antagonized by sulphate or complex nitrogenous supplements. The organism was readily 'trained' to resist this dye.

The control of the growth and metabolic activities of *Desulphovibrio desulphuricans* is a problem of practical as well as of theoretical interest, in view of their role both in the anaerobic corrosion of ferrous pipes (von Wolzogen Kuhr & van der Vlugt, 1934; Bunker, 1936, 1939; Hadley, 1943; Rogers, 1940, 1945; Starkey & Wight, 1945; Starkey, 1947; Butlin & Vernon, 1949; *Chemistry Research, 1938–46, 1947, 1948, 1949*) and in the pollution of water and sewage (Bunker, 1942; Rogers, 1945; Allen, 1949; Butlin, 1949). Hence the inhibition of these bacteria has been studied largely from a practical standpoint. Rogers (1940) investigated the resistance of crude cultures to various dyestuffs and observed that acriflavine and proflavine were their most active inhibitors. He studied in detail a dye of the acridinium group, '914', and showed that its activity depended on inoculum size. He noted the possibility that adaptation to resist the dyestuff could take place, and a further publication (Rogers, 1945) described the practical use of the dye. Bunker (1942) advocated decrease of pH value to control the pollution of relatively small water volumes by sulphate reducers. Allen (1949) confirmed this in a study of their activities in sewage, mentioned their high resistance to chlorine and showed that various nitro-compounds depressed H_2S formation. The resistance of these organisms to many common inhibitors and antibiotics has been studied (*Chemistry Research, 1947, 1948, 1949*), and an effect of ferrous ions in enhancing this resistance was mentioned (*Chemistry Research, 1947*). This effect could be partly due to differences between the methods used to detect growth in these experiments: blackening of the culture by ferrous sulphide formation can occur at levels of growth undetectable by direct inspection. Postgate (1949) described the competitive inhibition of sodium sulphate reduction by sodium selenate, using a non-growing cell suspension, and showed that sulphite overcame the consequent inhibition of hydrogen absorption in a non-competitive manner. The competitive inhibition of growth by selenate has been briefly reported, together with the failure of selenate to influence the reduction of thiosulphate and with manometric tests of selenite, tellurite and tellurate (*Chemistry Research, 1949*).

Fels & Cheldelin (1949*b*) showed that sodium sulphate overcame the inhibition of yeast growth by selenate in a competitive manner; with their organism methionine had some anti-selenate effect, though cysteine and glutathione had none (Fels & Cheldelin, 1948). In contrast, methionine had no anti-selenate effect for *Escherichia coli*, though cysteine and glutathione were active (Fels & Cheldelin, 1949*a*). Earlier studies by Hurd-Karrer (1937, 1938) on the selenate inhibition of growth of wheat plants showed that the effect of selenate depended on the amount of sulphate present, and that a molar proportion of selenate to sulphate considerably less than 1:1 prevented growth. She demonstrated that, at subinhibitory selenate levels, selenium was incorporated into the plant tissue in the same ratio to sulphur as in the nutrient solution provided.

The discovery of the anti-sulphate activity of selenate for *D. desulphuricans* led to a further investigation of this effect, together with a study of other sulphate analogues and some tests on the acridinium dye '914'. The results are reported here.

METHODS

Organism. A strain of *D. desulphuricans* called 'Hildenborough' was used throughout this work; its origin, maintenance and methods of subculture were described by Postgate (1951*a*).

Media. A medium (A) of: Bacto-tryptone (Difco) 5 g.; yeast extract (Difco) 4 g.; glucose 10 g.; $MgSO_4.7H_2O$ 1·5 g.; Na_2SO_4 1·5 g.; in 1 l. distilled water at pH 7·2 ± 0·2, was used to grow organisms for manometric work and for routine subculture. For growth experiments requiring media of known sulphate content a sulphate-deficient modification of this medium was used (medium B): Na_2SO_4 was omitted and $MgSO_4.7H_2O$ was replaced by $MgCl_2.6H_2O$ at 0·8 g./l. (Postgate, 1951*b*). Reagents were 'Analar' grade throughout except where mentioned in the text. The media containing glucose were sterilized by steaming for 1 hr. on three successive days; test solutions were autoclaved at 15 lb./sq.in. for 15 min. except for sodium sulphite, sodium thiosulphate and potassium chromate, which were sterilized by Seitz filtration. Potassium tellurate suspensions were not sterilized (below, p. 138).

Resting cell suspensions. Cell suspensions of 1–3 mg. dry wt./ml. in 0·8 % NaCl were prepared and, after pre-incubation in a stream of H_2 to remove traces of sulphate, 1 ml. portions were used in 2·5 ml. of liquid in the Warburg manometer at pH 6·3 as described by Postgate (1951*b*); cadmium chloride was used in the centre cup to absorb sulphide.

Growth tests. The effect of inhibitors on growth was studied by the conventional method of inoculating tubes of medium B containing a range of inhibitor concentrations and known amounts of Na_2SO_4. Media free of added iron were used since ferrous ions are known to alter the apparent sensitivity of sulphate-reducing bacteria to inhibitors.

Sulphate estimations. In one part of the work reported here the sulphate content of Warburg manometer suspensions was estimated turbidimetrically, after removal of bacteria, by treatment of 1 ml. of solution with 5 ml. of $BaCl_2$ (1 % w/v in 0·1 N-HCl) and diluting to 10 ml. final volume. This method was shown to estimate between 10^{-7} and 7×10^{-7} mol. Na_2SO_4 with no perceptible deviation among six tests, provided the precipitate was tested within 15 min. of preparation and provided the method was used only for the centrifuged residues of manometric experiments (below, p. 131).

RESULTS

Sodium selenate

The inhibition of sulphate reduction by selenate and the effect of sulphite have already been described (Postgate, 1949). In manometer vessels containing high concentrations of Na_2SO_4 and Na_2SeO_4 (British Drug Houses Ltd.) at a sub-inhibitory concentration ratio a red precipitate of selenium was found at the end of the experiment, the amount depending on the amount of selenate added.

Methionine, cysteine and glutathione, known to be anti-selenate substances for yeast and *Esch. coli* (Fels & Cheldelin, 1948, 1949*a*, *b*), were tested in the manometer at 1 mg./ml. as anti-selenate substances for *D. desulphuricans*. They did not affect the rate of sulphate reduction by partially inhibited cells (Table 1).

Table 1. *Hydrogen absorption by cell suspensions of* D. desulphuricans *(Hildenborough); test of various possible anti-selenate compounds*

Vessels contained 2·5 ml. of fluid at 37° C.

Na_2SO_4 (mol.)	Na_2SeO_4 (mol.)	Test substance (1 mg.)	Dry wt. of cells (mg.)	$-Q_{H_2}$ (mm.³/mg./hr.)
5×10^{-6}	—	—	0·74	361
5×10^{-6}	10^{-7}	—	0·74	108
5×10^{-6}	10^{-7}	Methionine	0·74	100
5×10^{-6}	10^{-7}	Glutathione	0·74	99
—	—	—	0·74	19
Another series:				
5×10^{-6}	—	—	1·18	530
5×10^{-6}	10^{-7}	—	1·18	84·5
5×10^{-6}	10^{-7}	Cysteine	1·18	86·5
—	—	—	1·18	16

The inhibition of hydrogen absorption by Na_2SeO_4 was overcome in a non-competitive manner by sodium sulphite, though preliminary experiments (Postgate, 1949) indicated that the reduction of Na_2SO_3 was in these circumstances incomplete. This finding suggested that selenate could block sulphate reduction at a second stage *en route* to sulphide. During this incomplete Na_2SO_3 reduction, however, sulphide was formed, and this observation was explained by the discovery of Na_2SO_4 as impurity in 'Analar' grade Na_2SO_3, the reduction of which was blocked by the Na_2SeO_4. The molecular weight of $Na_2SO_3.7H_2O$ (252) is fairly close to three-quarters of that of $Na_2SO_4.10H_2O$ (322), and since sulphite reduction absorbed 3 mol. H_2/ion and sulphate reduction 4 mol. H_2/ion, the presence of considerable amounts of hydrated Na_2SO_4 impurity remained undetected in manometric tests with Na_2SO_3 alone. 'Analar' grade Na_2SO_3 contained from 4 mol. % of Na_2SO_4 to as much as 75 mol. % of Na_2SO_4 in an old specimen; the sulphate could be removed by recrystallization of the less heavily contaminated batches.

Using pure Na_2SO_3 in the presence of Na_2SeO_4, 3 mol. H_2 were shown to be absorbed/SO_3'' ion reduced, and 1 mol. sulphide was found by iodometric analysis in the centre manometer cup (Postgate, 1951*b*). Results of an experiment demonstrating the complete reduction of Na_2SO_3 in the presence of concentrations of Na_2SeO_4 which inhibited Na_2SO_4 reduction are given in Table 2. A specimen of Na_2SO_3 contaminated with 30 mol. % of Na_2SO_4 was used, and the Na_2SO_4 remaining at the end of the experiment was found by centrifuging the suspension and estimating the sulphate turbidimetrically as $BaSO_4$. Controls were included to ensure that precipitation of $BaSeO_4$ did not interfere at the concentrations used.

Table 2. *Reduction of impure* Na_2SO_3 (*containing* 30 *mol.* % Na_2SO_4) *in the presence of* Na_2SeO_4 *by* D. desulphuricans (*Hildenborough*)

Vessels contained 2·5 ml. fluid at 37° and 2·84 mg. dry wt. cells.

Contents of vessel (mol. × 10^{-6})		Amount of H_2 absorbed (mol. % theoretical)	Amount of sulphide formed (mol. % theoretical)	Amount of sulphate remaining (mol. % of Na_2SO_3)
Na_2SO_3	Na_2SeO_4			
2·5	—	97	96	0
2·5	5	57	63	37
—	5	(Trace)	0	0

The reduction of sodium thiosulphate took place in the presence of Na_2SeO_4 concentrations which were able to prevent the reduction of ten times the molar concentration of Na_2SO_4. A cell suspension of 1·0 mg. dry wt. had a $-Q_{H_2}^{S_2O_3''}$ of 96 mm.^{3}/mg./hr. with $2·5 \times 10^{-6}$ mol. $Na_2S_2O_3$ and of 84 mm.^{3}/mg./hr. with $2·5 \times 10^{-6}$ mol. $Na_2S_2O_3 + 2·5 \times 10^{-6}$ mol. Na_2SeO_4. In manometric experiments using Na_2SO_3/Na_2SeO_4 and $Na_2S_2O_3/Na_2SeO_4$ mixtures, controls containing these mixtures and buffer without bacteria were included. After 90 min. incubation they were estimated for Na_2SO_3 or $Na_2S_2O_3$ iodometrically, and no losses of these compounds were detected. Thus there was no chemical reaction between selenate and the test materials at pH 6·3 and 37°.

Growth in medium B with 10^{-2} M-Na_2SO_4 was optimal, but a concentration of 10^{-1} M-Na_2SO_4 depressed growth (Postgate, 1951*b*). It was therefore undesirable to test the effect of Na_2SeO_4 on growth over a wide range of Na_2SO_4 concentrations, lest the depression of growth by strong Na_2SO_4 solutions should interfere with quantitative comparisons. Preliminary experiments showed that Na_2SeO_4 inhibited growth, and that at subinhibitory Na_2SeO_4 levels a red precipitate of selenium was formed. This precipitate interfered with accurate turbidimetric comparisons between cultures, but the tests gave an adequate indication that Na_2SeO_4 acted as a competitive Na_2SO_4 antagonist over a fivefold concentration range (Table 3). The molar Na_2SeO_4: Na_2SO_4 ratio required to prevent visible growth lay between 1:10 and 1 : 20, consistent with a ratio of between 1:10 and 1:15 found in manometric studies (Postgate, 1949).

Table 3. *Selenate-sulphate and selenate-sulphite antagonisms in growth of* D. desulphuricans (*Hildenborough*) *in medium B*

Cultures (5 ml.) were incubated for 120 hr. and then inspected.

Na_2SO_4 conc. (M)	Least conc. of Na_2SeO_4 preventing growth (M)	Greatest conc. of Na_2SeO_4 permitting growth (M)
10^{-2}	2×10^{-3}	10^{-3}
2×10^{-2}	4×10^{-3}	2×10^{-3}
5×10^{-2}	10^{-2}	5×10^{-3}
Na_2SO_3 conc. (M)		
10^{-2}	$>2 \times 10^{-2}$	$>2 \times 10^{-2}$

Table 3 also records that Na_2SO_3 acted as a non-competitive selenate antagonist in the growth of *D. desulphuricans*. Growth with 10^{-2} M-Na_2SO_3 occurred with $Na_2SO_3:Na_2SeO_4$ ratios from 10:1 to 1:2, above which they were not tested. The presence of Na_2SeO_4 decreased the rate and extent of growth at all ratios, the cultures containing Na_2SeO_4 growing about 24 hr. later than the controls and to about half the extent. Useful quantitative measurements of this effect could not be obtained owing to a precipitate of selenium which appeared in the cultures once the logarithmic phase of growth had started. Controls containing Na_2SO_3 and Na_2SeO_4 were set up without inoculation, and iodometric analysis of these after incubation showed that no chemical reaction between selenate and sulphite had occurred. The effect of $Na_2S_2O_3$ on growth in selenate-containing media was not studied since, after incubation at 30° for 16 hr., chemical reduction of Na_2SeO_4 giving a precipitate of selenium occurred even in uninoculated cultures.

Since the formation of selenium in growing cultures containing Na_2SeO_4 might have represented a mechanism whereby cells could detoxify their medium, an attempt was made to obtain a strain able to resist Na_2SeO_4. After twelve subcultures with 10^{-2} M-Na_2SO_4 and subinhibitory Na_2SeO_4

concentrations the strain had undergone no increase in its resistance to Na_2SeO_4, and the attempt was abandoned.

Potassium tellurate

The formula of potassium tellurate is sometimes written as $K_2TeO_4 . 2H_2O$, but it is now known to be a derivative of *ortho*telluric acid ($Te(OH)_6$) rather than of the hypothetical *meta*telluric acid (H_2TeO_4). Hence it is best represented as $K_2H_4TeO_6$; its structural resemblance to K_2SO_4 is consequently remote. Moreover, the alkali *ortho*tellurates do not form real solutions, but dissolve as 'semi-colloidal' suspensions which are optically inhomogeneous (Sidgwick, 1950); it is therefore unlikely that significant amounts of the strict sulphate analogue, the TeO_4'' ion, exist in solutions of these salts. It was nevertheless thought desirable to test a tellurate. Potassium tellurate (British Drug Houses Ltd.) was used and assumed to have a molecular weight of 360, corresponding to its most common hydrate, $K_2H_4TeO_6 . 3H_2O$. 'Semi-colloidal' suspensions of this salt were destroyed by heat sterilization, and a 1/10 dilution of the clear supernatant did not affect growth or sulphate reduction, so all growth tests were performed with unsterilized tellurate suspensions. Cultures in which growth occurred were examined for contaminants, but none were found.

A cell suspension of 1·12 mg. dry wt. in the Warburg manometer reduced $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 with a $-Q_{H_2}^{SO_4''}$ of 202 mm.³/mg./hr., and this value was unaffected by 5×10^{-7} and by 10^{-6} mol. of potassium tellurate as a suspension. The value of $-Q_{H_2}^{SO_4''}$ was reduced to zero by 2×10^{-6} mol. suspension and this effect was not prevented by increasing the sulphate concentration tenfold to $2{\cdot}5 \times 10^{-5}$ mol. Na_2SO_4. In medium B growth after 168 hr. with 10^{-2}M-Na_2SO_4 was prevented by a 2×10^{-5}M tellurate suspension but was permitted by a 10^{-5}M suspension; a fivefold increase in the Na_2SO_4 concentration to 5×10^{-2}M did not affect these figures. Hence, though potassium tellurate inhibited both sulphate reduction and growth, this inhibition was not by way of competitive sulphate antagonism.

Ammonium monofluorophosphate

The monofluorophosphate ion, PO_3F'', is analogous to sulphate in both geometry and electron distribution; monofluorophosphates have many properties in common with sulphates (Ephraim, 1934). Ammonium monofluorophosphate, $(NH_4)_2PO_3F . H_2O$, prepared by the method of Lange (1929), was not reduced by resting cell suspensions of *D. desulphuricans*. The reduction of $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 was prevented by $3{\cdot}75 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$, i.e. a molar ratio $PO_3F'' : SO_4''$ of 3:2; at a lower $PO_3F'' : SO_4''$ ratio (2:5) partial inhibition occurred. This inhibition was competitive over a tenfold range of substrate and inhibitor concentrations, as the rate of hydrogen absorption was determined by the molar ratio of $PO_3F'' : SO_4''$ and not by their absolute amounts (Fig. 1). At higher levels (10^{-4} mol. $(NH_4)_2PO_3F + 2{\cdot}5 \times 10^{-4}$ mol. Na_2SO_4) complete inhibition of sulphate reduction was found, though the inhibitor:substrate ratio was still 2:5. This non-competitive inhibition may have been a secondary toxic action of $(NH_4)_2PO_3F$, or merely attributable to the high salt concentrations in the test media; it was not studied further.

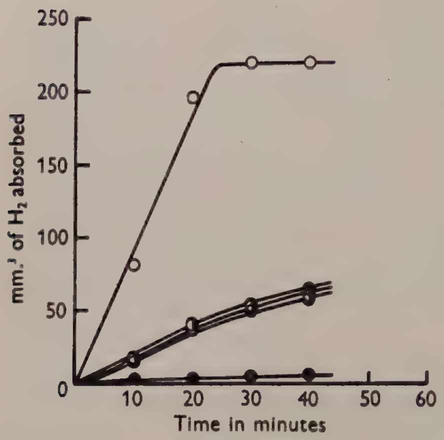

Fig. 1

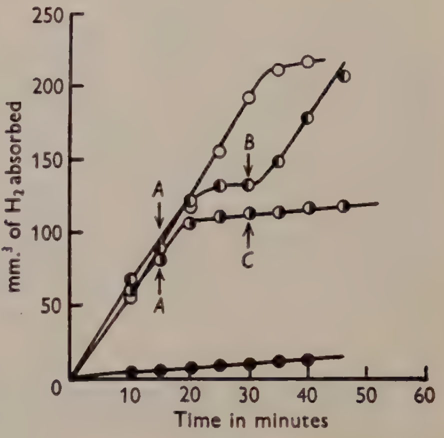

Fig. 2

Fig. 1. Competitive antagonism of sulphate reduction by monofluorophosphate with *D. desulphuricans* (Hildenborough). 3·84 mg. dry wt. cells/manometer vessel. ○, $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4; ◒, 5×10^{-6} mol. $Na_2SO_4 + 2 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$; ◓, 10^{-5} mol. $Na_2SO_4 + 4 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$; ◑, $2{\cdot}5 \times 10^{-5}$ mol. $Na_2SO_4 + 10^{-5}$ mol. $(NH_4)_2PO_3F$; ●, no substrate.

Fig. 2. Non-competitive monofluorophosphate antagonism by sulphite with *D. desulphuricans* (Hildenborough). 2·74 mg. dry wt. cells/manometer vessel. ○, $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4; ●, no substrate; ◐, $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 added at start, then $3{\cdot}75 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$ at *A*, followed by $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_3 at *B*; ◑, $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 added at start, then $3{\cdot}75 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$ at *A*, followed by $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 at *C*.

Sulphite was a non-competitive antagonist of the inhibition of hydrogen absorption caused by $(NH_4)_2PO_3F$ in the presence of Na_2SO_4. The absorption of hydrogen consequent on the reduction of $2{\cdot}5 \times 10^{-6}$ mol. Na_2SO_4 was prevented by addition of $3{\cdot}75 \times 10^{-6}$ mol. $(NH_4)_2PO_3F$. Addition of a molar concentration of Na_2SO_3 equal to that of the Na_2SO_4 originally present permitted the resumption of hydrogen absorption, whereas addition of an equivalent molar Na_2SO_4 concentration did not (Fig. 2). There was a time lag of several minutes after addition of $(NH_4)_2PO_3F$ before sulphate reduction ceased, suggesting that the PO_3F'' ion was slow to penetrate the cell and reach the sulphate-reducing enzyme. No such lag was observed with Na_2SeO_4. $Na_2S_2O_3$ was also a non-competitive $(NH_4)_2PO_3F$ antagonist, being reduced in the presence of sufficient $(NH_4)_2PO_3F$ to prevent the reduction of 8 times the molar concentration of Na_2SO_4.

$(NH_4)_2PO_3F$ also acted as a competitive sulphate antagonist in the growth of *D. desulphuricans* (Table 4); a molar $PO_3F'':SO_4''$ ratio between 2:1 and 1:1 was needed to prevent visible growth in medium B. Half growth occurred with a $PO_3F'':SO_4''$ ratio between 1:1 and 1:2. Table 4 also records that both

Na_2SO_3 and $Na_2S_2O_3$ acted as non-competitive antagonists of $(NH_4)_2PO_3F$. Growth with these substrates occurred at all $(NH_4)_2PO_3F$ concentrations tested; at the highest test concentration, 10^{-1} M, growth was delayed in both cases.

Table 4. *Monofluorophosphate-sulphate antagonism in growth of* D. desulphuricans (*Hildenborough*) *in medium B*

Cultures (5 ml.) were incubated for 162 hr. and inspected.

Na_2SO_4 conc. (M)	Least conc. of $(NH_4)_2PO_3F$ preventing growth (M)	Greatest conc. of $(NH_4)_2PO_3F$ permitting growth (M)
10^{-2}	2×10^{-2}	10^{-2}
2×10^{-2}	4×10^{-2}	2×10^{-2}
5×10^{-2}	10^{-1}	5×10^{-2}
Na_2SO_3 conc. (M)		
10^{-2}	$>10^{-1}$	$>10^{-1}$
$Na_2S_2O_3$ conc. (M)		
10^{-2}	$>10^{-1}$	$>10^{-1}$

Potassium chromate

An early report (*Chemistry Research, 1948*) that K_2CrO_4 inhibited the growth of sulphate-reducing bacteria, and that its effect was antagonized by ferrous ions, did not discuss whether it was a sulphate antagonist, though the chromates and sulphates are analogues in Group VI of the Periodic Table.

In the Warburg manometer K_2CrO_4 was a powerful inhibitor of sulphate reduction, and $2{\cdot}5\times10^{-7}$ mol. K_2CrO_4 prevented the reduction of $2{\cdot}5\times10^{-6}$ mol. Na_2SO_4 and of $2{\cdot}5\times10^{-5}$ mol. Na_2SO_4. Partial inhibition of sulphate reduction was obtained by $2{\cdot}5\times10^{-8}$ mol. K_2CrO_4. The rate of sulphate reduction in these conditions was independent of the sulphate concentration over a tenfold range (Table 5).

Table 5. *Effect of chromate on reduction of sulphate. Hydrogen absorption by cell suspensions of* D. desulphuricans (*Hildenborough*)

Vessels contained 2·5 ml. fluid at 37° and 2·16 mg. dry wt. cells

Na_2SO_4 (mol. $\times 10^{-6}$)	K_2CrO_4 (mol. $\times 10^{-6}$)	$-Q_{H_2}$ (mm.^{3}/mg./hr.)
2·5	—	490
2·5	0·025	276
25	0·025	276
2·5	0·25	0
25	0·25	0
—	—	5·5

Experiments on the growth in medium B are reported in Table 6. Visible growth with 10^{-2} M-Na_2SO_4 was prevented by K_2CrO_4 concentrations in the region of 10^{-4} M. This figure was unaltered by a fivefold increase in the sulphate concentration; thus chromate was not a competitive antagonist of sulphate reduction.

Table 6. *Inhibition by chromate of growth of* D. desulphuricans (*Hildenborough*) *in medium B*

Cultures (5 ml.) were incubated 144 hr. and inspected.

Na_2SO_4 conc. (M)	Least conc. of K_2CrO_4 preventing growth (M)	Greatest conc. of K_2CrO_4 permitting growth (M)
10^{-2}	10^{-4}	5×10^{-5}
5×10^{-2}	10^{-4}	5×10^{-5}

Other analogues of sulphate

The perchlorate ion and its homologues in Group VII of the Periodic Table are in a broad sense analogues of the sulphate ion, though they have only one charge. As an example of this group potassium perchlorate ($KClO_4$, British Drug Houses Ltd.) was tested. Other analogues of sulphate used here involved the replacement of oxygen atoms in the ion by basic groups, and were of three main classes: (i) alkyl and aryl sulphonic acids, in which one oxygen atom is replaced, e.g. sodium methanesulphonate ($NaO.SO_2CH_3$; synthetic), sodium β-hydroxyethanesulphonate ($NaO.SO_2CH_2CH_2OH$; synthetic), and sodium benzenesulphonate ($NaO.SO_2\phi$; British Drug Houses Ltd.); (ii) sulphones, in which two oxygen atoms are substituted, e.g., dimethylsulphone ($(CH_3)_2SO_2$; synthetic); (iii) compounds such as sulphamic acid and nitrosylsulphuric acid, in which an oxygen atom is exchanged for a basic inorganic group; represented by ammonium sulphamate ($NH_4.SO_3NH_2$; Baird and Tatlock Ltd.). Partial esterification of the sulphate ion gives compounds having a structural resemblance to sulphate; as an example sodium ethylsulphate ($Na(C_2H_5)SO_4$; synthetic) was tested.

All these compounds were tested with resting suspensions in the Warburg manometer in equimolecular proportion to the Na_2SO_4 added; $2 \cdot 5 \times 10^{-6}$ mol. of substrate and test material were used. In no case was the rate of sulphate reduction affected by these materials (Table 7), so the findings were checked by testing these compounds at ten times the equivalent molar sulphate concentration, using $2 \cdot 5 \times 10^{-6}$ mol. Na_2SO_4 and $2 \cdot 5 \times 10^{-5}$ mol. test material. The only compound to show activity in these tests was $KClO_4$, which depressed the reduction rate by about 85 %. The diminished rate of reduction was independent of the Na_2SO_4 concentration over a tenfold range, however, indicating that this was not competitive sulphate antagonism (Table 7).

Acridinium dye 914

Since dye 914 (Imperial Chemical Industries Ltd.) has a growth-inhibitory effect on *D. desulphuricans* in crude cultures (Rogers, 1940, 1945) it was desirable to find out whether it acted by interference with sulphate metabolism. A concentration of dye about ten times that required to prevent growth, however, did not significantly affect sulphate reduction. A cell suspension of 2·9 mg. dry wt. reduced $2 \cdot 5 \times 10^{-6}$ mol. Na_2SO_4 with a $-Q_{H_2}^{SO_4}$ of 236 mm.³/mg./hr.; with 250 μg. dye the quotient was 212 mm.³/mg./hr.

Table 7. *Tests of various potential inhibitors of sulphate reduction. Hydrogen absorption by cell suspensions of* D. desulphuricans (*Hildenborough*)

Vessels contained 2·5 ml. fluid at 37°.

Na_2SO_4 (mol. × 10^{-6})	Compound tested (mol. × 10^{-6})	Cells (mg. dry wt.)	$-Q_{H_2}$ (mm.³/mg./hr.)
2·5	—	2·2	210
2·5	$NaO.SO_2.CH_3$; 2·5	2·2	202
2·5	$NaO.SO_2.C_2H_4OH$; 2·5	2·2	205
2·5	—	2·4	180
2·5	$NH_4SO_3NH_2$; 2·5	2·4	180
2·5	$NaO.SO_2\phi$; 2·5	2·4	180
2·5	—	1·84	260
2·5	$(CH_3)_2SO_2$; 2·5	1·84	260
2·5	—	1·0	735
2·5	$(CH_3)_2SO_2$; 25	1·0	790
2·5	$Na(C_2H_5)SO_4$; 25	1·0	732
2·5	—	0·67	331
2·5	$HN_4.SO_3NH_2$; 25	0·67	339
2·5	$NaO.SO_2\phi$; 25	0·67	325
2·5	—	0·59	570
2·5	$NaO.SO_2.CH_3$; 25	0·59	580
2·5	—	1·62	310
2·5	$NaO.SO_2.C_2H_4OH$; 25	1·62	320
2·5	$KClO_4$; 25	1·62	59
25	$KClO_4$; 25	1·62	61
—	—	1·62	2

The inhibitory effect of dye 914 on growth was also unaffected by the Na_2SO_4 concentration. Visible growth was prevented by between 8 and 10 μg. dye/ml. in medium B containing either 10^{-2} M-Na_2SO_4 or five times this amount. Postgate (1951*a*) showed that peptone and yeast extract contained growth-promoting substances for this strain of *D. desulphuricans*, but the resistance of cells to dye 914 was not affected by the presence or absence of these supplements. The resistance of the cells remained at *c.* 10 μg. dye/ml. whether they were grown in medium A or in Starkey's (1938) medium containing only lactic acid and inorganic salts.

The possibility, mentioned by Rogers (1940), that dye-resistant strains might appear in low concentrations of dye 914 was confirmed. After six passages in medium A with subinhibitory amounts of dye the resistance had increased at least twenty-fold (Table 8). As in all this work, the inoculum size was 1 μg. dry wt. cells/5 ml. culture at each subculture.

DISCUSSION

The findings with dye 914 require little discussion. Its inhibitory action was not correlated with the peculiar metabolism of *D. desulphuricans*; it was not demonstrably related to the process of sulphate reduction and was unrelated to the need of this organism for certain substances in peptone and yeast extract. Resistance to the dye was acquired readily, but there is no evidence from this work to indicate whether the process was 'adaptational' or 'mutational'.

It is now well known that structural analogues of metabolites often inhibit the utilization of metabolites in a competitive manner. Since the first demonstration of this point (Woods, 1940) the concept of metabolite antagonism has been widely applied in the search for inhibitors of therapeutic value. Metabolite analogues have most often been selected to antagonize the utilization of growth factors or amino-acids: substances required only in small amounts but whose presence is essential. Thus the growth of an organism can be prevented by small amounts of this kind of anti-metabolite. In the present case, however, sulphate is required by *D. desulphuricans* in relatively large amounts since sulphate reduction is the main energy-yielding reaction for growth. The competitive inhibition of sulphate-hydrogenase by selenate and monofluorophosphate is thus more strictly analogous to the inhibition of a catabolic reaction, such as the competitive inhibition of succinic dehydrogenase by malonate (Hopkins, Morgan & Lutwak-Mann, 1938), rather than to the inhibitions involving competition with trace factors. Woods (1949) pointed out that competitive antagonism suggests that the anti-metabolite inhibitor is adsorbed at the surface of the enzyme which uses the metabolite, and that a substance which overcomes the effect of the inhibitor in a non-competitive manner may represent one of the normal products of the inhibited reaction. Nimmo-Smith, Lascelles & Woods (1948) showed that this occurred with *Streptobacterium plantarum*: pteroylglutamic acid was a non-competitive sulphanilamide antagonist and sulphanilamide interfered competitively with its synthesis from *p*-aminobenzoic acid by the organism. The effect of sulphite in overcoming the inhibitory effects of selenate and monofluorophosphate on *D. desulphuricans*, reported here, was probably a similar instance; sulphite was probably a normal intermediate in the reduction of sulphate. On the other hand, the case of thiosulphate is not so clear, since, though it was a non-competitive antagonist of selenate and monofluorophosphate inhibition, there is evidence, from the rate at which cells reduce it, that it is not a normal intermediate in sulphate reduction (Postgate, 1951*b*). It is possible that it acted as a non-competitive antagonist because it was reduced by a mechanism different from normal sulphate reduction.

Table 8. '*Training*' D. desulphuricans (*Hildenborough*) *to resist dye* 914 *in medium B*

Serial no. of subculture	Interval between subcultures (days)	Highest concentration of dye permitting growth (μg./ml.)	Lowest concentration of dye preventing growth (μg./ml.)
1	—	8	10
2	4	8	10
3	9	10	12
4	12	12	—
5	7	16	—
6	3	100	200

That the sulphur-containing compounds methionine, cysteine and glutathione had no anti-selenate effect with *D. desulphuricans* contrasts with the findings of Fels & Cheldelin (1948, 1949*a*, *b*) with yeast and *Esch. coli* already described. The difference in behaviour is probably attributable to the fact that sulphate-reduction is quantitatively most important as an energy-yielding reaction for *D. desulphuricans*. The reactions corresponding to those studied by Fels & Cheldelin, leading to the synthesis of sulphur containing amino-acids and peptides, play a quantitatively small part in the sulphate metabolism of this genus, and contribute negligibly to the total turnover of sulphate.

Competitive metabolite antagonism is often pictured as a simple physico-chemical process in which no change occurs to the inhibitor; it merely displaces the metabolite from its enzyme, and both thermodynamic and kinetic treatments of competitive interrelations have been put forward based on this assumption (Wyss, 1941; Klotz, 1944; Klotz & Gutmann, 1945). However, Hurd-Karrer (1937, 1938) showed that selenium was incorporated in the tissues of partially selenate-inhibited wheat plants, and in the present work there is little doubt that selenate at subinhibitory concentrations was reduced to selenium, though this reduction did not interfere with the competitive nature of selenate inhibition. It is unlikely that the normal sulphate-utilizing enzyme was involved in selenate reduction, since deposition of selenium occurred only at levels of selenate which caused partial inhibition; no deposition of selenium by completely inhibited cells was ever observed, either in growth or manometric experiments. Moreover, if selenate were attacked directly, it is curious that the 'training' process to which the organisms were subjected did not give rise to a strain better able to reduce selenate and thus of greater selenate resistance. Selenate is readily reduced by chemical means, and it is probable that the deposition of selenium was a side-reaction involving the reaction of selenate with some reducing agent produced by actively metabolizing cells.

The activity of a metabolite analogue depends not only on its steric resemblance to the metabolite, but also on its resemblance in electron distribution. Bell & Roblin (1942) correlated the inhibitory action of the sulphonamide drugs with their electronic resemblance to *p*-aminobenzoic acid as reflected in their acid dissociation constants. By a study of anti-tryptophan substances in which the double bonds of the aromatic indole system were stabilized in various positions, Fildes & Rydon (1947) obtained strong indications of the form in which the tryptophan molecule combined with its utilizing enzyme in *Bact. typhosum*. Inspection of the structures of the compounds tested in the present work (Fig. 3) shows that two charges on the ion were necessary for interaction with the sulphate-utilizing enzyme; all the singly-charged sulphate analogues were unable to affect the enzyme. Moreover, provided the requirements of charge distribution were satisfied, an ion such as PO_3F'', which has an atomic constitution very different from sulphate, was able to interact with the sulphate-utilizing enzyme. The inactivity of tellurate as a sulphate antagonist may be attributed to the fact that the TeO_4'' ion was probably unstable in the test conditions.

There is no obvious reason why chromate should have been inactive, as it satisfies the requirement for charge, and its general shape—a symmetrical tetrahedral ion—resembles that of sulphate. It had, however, a strong non-specific toxic effect on cells of *D. desulphuricans* even at low concentrations, and that may have masked any competitive activity. Alternatively, as the action of monofluorophosphate on sulphate reduction was delayed, presumably owing to slow penetration of inhibitor to enzyme, and since the chromate ion was the largest of the simple divalent ions tested, chromate may have been too large to reach the enzyme at all. The diameters of the sulphate, selenate and monofluorophosphate ions are similar, whereas the diameter of the chromate ion is considerably greater.

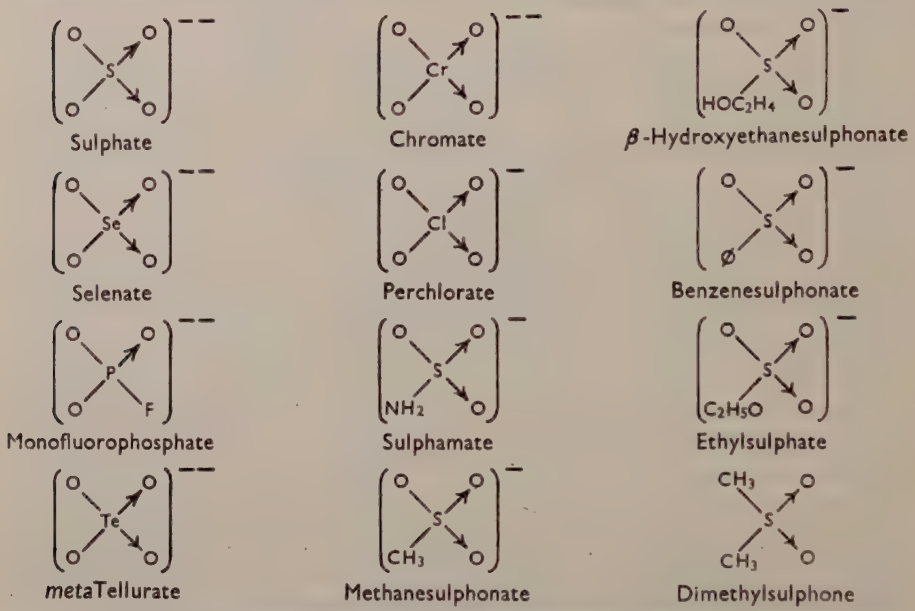
Sulphate
Chromate
β-Hydroxyethanesulphonate
Selenate
Perchlorate
Benzenesulphonate
Monofluorophosphate
Sulphamate
Ethylsulphate
*meta*Tellurate
Methanesulphonate
Dimethylsulphone

Fig. 3. The chemical structures of some analogues of the sulphate ion.

These studies have mainly been concerned with antagonism of the utilization of an inorganic anion by other anions; most studies in the field of metabolite antagonism have been restricted to antagonists of complex organic molecules, though Macleod & Snell (1948) demonstrated antagonisms between alkali metal cations in the growth of lactic bacteria. The molar concentration ratios permitting a half-maximal rate of sulphate reduction illustrate the remarkably high specific activities of the two competitive inhibitors studied here. For the antagonism of *p*-aminobenzoic utilization by sulphonamides, for example, molar concentrations several hundred times that of the *p*-aminobenzoic acid may be required for half-inhibition, and even with the highly active folic acid antagonist, aminopterin, a molar ratio of inhibitor to metabolite less than 1:1 is rare, though it occurs with *Streptococcus faecalis* R at high concentrations of metabolite and antagonist (Franklin, Belt, Stokstad & Jukes, 1949). In the present work, however, a $Na_2SeO_4:Na_2SO_4$ ratio of 1:10 completely inhibited sulphate reduction, one of about 1:50 permitted a half-maximal reduction rate, and an $(NH_4)_2PO_3F:Na_2SO_4$ ratio of about 2:5 gave a half-maximal rate. Selenate thus showed a remarkably high specific activity, and though the activity of monofluorophosphate was but a twentieth of this, it was nevertheless high for a competitive metabolite antagonist. It is possible that selenate and monofluorophosphate are concentrated inside the cell by conversion to a non-diffusible active form, thus becoming highly active at their site of action. On the other hand, if the effect of these inhibitors was to interfere with sulphate reduction by a simple physico-chemical process, by competitive reaction with or adsorption on the sulphate-reducing enzyme, then this enzyme must have had a greater chemical affinity for the inhibitors than for its normal substrate. The free energy of formation of the inhibitor-enzyme complex must have been greater, and in the case of selenate, very much greater, than the free energy of formation of the sulphate-enzyme complex.

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Observations on the Relationship between Viruses of the Psittacosis-Lymphogranuloma Group and the Rickettsiae

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SUMMARY: Changes in the metabolism of the chick embryo host cell produced by *p*-aminobenzoic acid or sodium cyanide did not influence the multiplication of psittacosis or Newcastle virus. A suspension of psittacosis virus was unable to catalyse oxygen uptake in presence of glutamic acid, casein hydrolysate, succinic or pyruvic acids. The results suggest that, in its relation to certain metabolic activities of the host cell, psittacosis virus more nearly resembles the true viruses than the *Rickettsiae*.

The systematic position of the psittacosis-lymphogranuloma group of infective agents is still a matter of dispute. Some authorities, impressed by the resemblances of these agents to the Rickettsiae, have suggested that they should be grouped with them. Lillie (1930), who was one of the first to make a morphological study of psittacosis virus, stated that he considered it to be a rickettsia, naming it *Rickettsia psittaci*; others retain these agents with the viruses. A third view is that they form a group by themselves, occupying a position intermediate between the rickettsiae and what are termed the 'true viruses'. Admittedly the agents of the psittacosis-lymphogranuloma group resemble the rickettsiae in some respects. They have a similar affinity for basic dyes which appears to be related to their high content of deoxyribonucleic acid (Lépine & Sautter, 1946). Although, in general, they are smaller than the rickettsiae they are not dissimilar in this respect from *R. burneti*. They produce intracellular colonies which resemble those of the rickettsiae rather than the inclusions of the true viruses, and their susceptibility to sulphonamides and some natural antibiotics, though differing in some respects from that of the rickettsiae, suggests a closer resemblance to the latter than to the viruses. There are, however, points of difference. The agents of the psittacosis-lymphogranuloma group do not appear in bacillary form as do the rickettsiae, and when multiplying they pass through a cycle of development not seen in the rickettsiae. A more important dissimilarity is the difference in host range: the rickettsiae are essentially parasites of arthropods, though some species have also acquired mammalian hosts, whereas none of the viruses of the psittacosis-lymphogranuloma group has an insect host. The recent work of Greiff & Pinkerton (1948) may well have a bearing on the question of the relationship of the psittacosis-lymphogranuloma group to the viruses on the one hand, and the rickettsiae on the other. These workers found that injection of *p*-aminobenzoic acid (PAB) into the developing hen's egg increased the metabolism of the cells of the embryo as measured by respiratory exchange, making them in consequence unsuited to the multiplication of typhus rickettsiae; potassium cyanide had the reverse effect. Zinsser & Schoenbach (1937) claimed that an essential difference between rickettsiae and viruses is that the former begin to multiply in cells when cell metabolism has diminished, whereas viruses require for multiplication cells at the height of their metabolic activity. In the light of these observations it seemed of importance to repeat the work of Greiff & Pinkerton with a virus of the psittacosis-lymphogranuloma group. Similarly, the investigations of Bovarnick & Snyder (1949) showing the ability of typhus rickettsiae preparations to catalyse the uptake of oxygen in the presence of certain substrates, in particular glutamic acid, have a bearing on this problem and it was thought of interest to see whether a virus of the psittacosis-lymphogranuloma group behaved in a similar manner. Psittacosis virus was chosen for this purpose, with Newcastle disease virus, representing the true viruses, as a control.

Effect of p-*aminobenzoic acid and of sodium cyanide on the growth of psittacosis virus in the yolk sac of the developing chick embryo*

Psittacosis virus. Strain MOH 154 (isolated from a parrot in 1940; maintained in the mouse until 1945; established in eggs after that date); material from the 146th to the 158th egg passage was used.

Chick embryos. Fertile eggs were incubated at 39° before inoculation. After inoculation they were incubated at 35° until harvested.

p-*Aminobenzoic acid.* Aqueous solution, 16 mg./ml.; dose used 0·2 ml.

Sodium cyanide. Aqueous solution, 0·5 mg./ml.; dose used 0·2 or 0·4 ml. Both these solutions were sterilized by means of sintered glass filters (porosity L 5).

Technique. The PAB or sodium cyanide solution was injected into the air sac of the embryo (in some experiments into the yolk sac). Preliminary experiments, using methylene blue or estimations of PAB in various parts of the embryo, established that rapid diffusion of electrolytes across the shell membrane into all parts of the embryo occurred. Where double inoculation of eggs was necessary, injection into the air sac diminished trauma to a negligible degree. PAB was always introduced 48 hr. before virus since, as Greiff & Pinkerton showed, the maximum effect on metabolism of the embryo occurs on the fourth day following inoculation. Furthermore, even dilute suspensions of psittacosis virus killed embryos in approximately 4 days, so that if virus and PAB had been injected simultaneously there would have been insufficient time for the maximum effect. In contrast, sodium cyanide has an almost immediate effect on metabolism and so could be inoculated at the same time as the virus. Virus was inoculated direct into the yolk sac. In certain experiments both virus and PAB or sodium cyanide were injected directly into the yolk sac.

In each experiment two series of embryos were used: a control series received saline injected into the air sac, and the test series received a dose of PAB or sodium cyanide. Both series were then injected with an equal dose of psittacosis virus. On the fourth day following infection the majority of embryos in both series were dead or moribund. All yolk sacs were harvested into normal saline, one yolk sac/2 ml. saline. Embryos dying on the first or second day following infection with virus were discarded as non-specific deaths.

Estimation of virus growth. Individual yolk sac impression preparations were made and stained by Castaneda's method. All the yolk sacs in each group were then pooled, ground in a Tenbroeck tube, and after sedimentation overnight in the refrigerator, smears were made from the supernatant fluids and compared. A crude system of marking from ± to ++++ was then accorded to the virus content of these preparations.

Results with psittacosis virus

Neither PAB nor cyanide in the concentrations used influenced the rate of multiplication of psittacosis virus in the yolk sac of the developing chick embryo. Since impression preparations of individual yolk sacs showed considerable variation, a variation which might well be due to chance, only the findings in the smears made from pooled suspensions are recorded. Thus when PAB (3·3 mg./sac) was injected into the yolk sac of ten embryos the smear of the pool scored ++ amounts of virus, the same as the score of the pool of nine control embryos. Similarly, the scores were equal in the pool of eight embryos injected with PAB in the air sac and the eight-embryo control pool. Sodium cyanide (0·2 mg./air sac) gave a score of ++ in an eight-embryo pool and the same for a nine-embryo control pool; 0·1 mg. NaCN/yolk sac gave + in test pool (9) and also + in control pool (7). There is nothing in these results to suggest that either PAB or cyanide influenced the growth of psittacosis virus, a finding supported by the fact that approximately equal numbers of embryos in the control and experimental series were dead or moribund on the fourth day.

Effect of p-*aminobenzoic acid and sodium cyanide on the growth of Newcastle virus in the chorio-allantoic membrane of the developing chick embryo*

Newcastle virus. An egg-adapted strain received from Mr T. M. Doyle, F.R.C.V.S. was used. Saline suspensions of freshly harvested infected allantoic fluid (10 to 12-day embryos) or of reconstituted freeze-dried material were used in dilutions of 10^{-5} in an inoculum of 0·1 ml. As far as possible the minimum infecting dose was established for each preparation by titration in embryos of the same age as those used in the actual experiments.

Chick embryos. Ten to twelve-day embryos were used, the temperatures of incubation being the same as those used for the psittacosis experiments.

Technique of procedure. This was like that used with psittacosis virus except that Newcastle virus was injected by the allantoic route. Allantoic fluids were harvested from both series at the end of 48 hr. and their virus content estimated by haemagglutination.

Haemagglutination. The Salk (1944) method was followed, using either fowl or human red cells. Results were read after allowing sedimentation of cells at +4° for 6 hr. The end-point titre was taken as the reciprocal of highest dilution in which red cells were completely dispersed on the bottom of the tube without ring formation. A pro-zone, probably due to interference by yolk, was met in some titrations; where this occurred in association with a low haemagglutinin titre a clear end-point was not always obvious. Since this phenomenon occurred in both groups with approximately equal frequency it did not invalidate comparison between the two.

Results with Newcastle virus

As with psittacosis virus, neither cyanide nor PAB could be shown to influence the rate of multiplication of Newcastle virus in allantoic fluid as estimated by haemagglutination (Tables 1 and 2). In Exp. 3 (Table 2) some embryos in both groups were alive at the time of harvesting and no virus was detected. This was presumed to be due to the infecting dose of virus having been a minimum infecting dose. It was the deliberate aim in these experiments to keep the infecting dose at this level in order to reveal any small influence which cyanide or PAB might have on the growth of the viruses.

Table 1. *The effect of PAB on the growth of Newcastle virus in the chick embryo*

PAB injected by air sac route. Virus estimated by agglutination of fowl red cells.

Dose of PAB (mg.)	Virus inoculum	PAB series		Control series	
		No. of embryo	Titre	No. of embryo	Titre
		Exp. 1			
6·6	Freeze dried material; 0·1 ml. of 10^{-5} dilution	1	5,120	1	10,240
		2	40,960	2	10,240
		3	10,240	3	20,480
		4	10,240	4	10,240
		5	10,240	5	—
		6	5,120	6	10,240
		7	5,120	7	20,480
		Exp. 2			
3·3	Freeze dried material; 0·1 ml. of 10^{-5} dilution	1	5,120	1	2,560
		2	160	2	—
		3	10,240	3	10,240
		4	320	4	10,240
		5	—	5	10,240
		6	2,560	6	10,240
		7	40 (*i*)	7	10,240
		8	10,240	8	5,120
		9	20,480	9	10,240
		10	*d*	10	5,120

d=eggs dead after 24 hr. and discarded; — =no virus detected; (*i*)=incomplete dispersion of red cells at end point.

Inability of psittacosis virus to catalyse oxidation of certain substrates

The technique used was that described by Bovarnick & Snyder (1949), and respiratory exchange was measured in the conventional Warburg apparatus. Psittacosis virus was harvested in the same buffer as that used by these authors, but the method of preparing the virus suspension differed from their technique

Table 2. *The effect of sodium cyanide on the growth of Newcastle virus in chick embryo*

Sodium cyanide injected by air sac route. Virus estimated by agglutination of human red cells.

Dose of sodium cyanide (mg.)	Virus inoculum	Sodium cyanide series		Control series	
		No. of embryo	Titre	No. of embryo	Titre
		Exp. 1			
0·2	Freeze dried material; 0·1 ml. of 10^{-5} dilution	1	160	1	160
		2	40 (*i*)	2	160 (*i*)
		3	40	3	320
		4	—	4	320
		5	80	5	—
		6	40	6	1280
		7	160	7	80
		Exp. 2			
0·2	Fresh allantoic fluid; 0·1 ml. of 10^{-5} dilution	1	2560	1	320
		2	—	2	—
		3	2560	3	1280
		4	5120	4	320
		5	1280	5	—
		6	*d*	6	320
		7	*d*	7	2560
		Exp. 3			
0·1	Fresh allantoic fluid; 0·1 ml. of 10^{-5} dilution	1	320	1	20
		2	320	2	320
		3	160	3	640
		4	2560	4	320
		5	320	5	80
		6	1280	6	160
		7 8 9 10	(Alive)	7	320
				8	640
				9 10	(Alive)

Symbols as in Table 1.

with rickettsiae. Infected yolk sacs from 8- to 9-day chick embryos were harvested in the proportion of one yolk sac/2·5 ml. buffer (pH 7·5). The intact yolk sacs were gently shaken and the suspensions centrifuged at 2500 r.p.m. for 15 min. The supernatant was pipetted off, care being taken not to disturb the surface disk of yolk and fat. By this method a rich yield of virus containing few, if any, cells was obtained. More violent methods of triturating yolk sacs released cellular material without greatly increasing the virus content.

Examination for oxygen uptake in Warburg apparatus. All experiments were performed at a water-bath temperature of 36°. The mixture in each Warburg flask consisted of: 1·5 ml. psittacosis virus suspension; 0·2 ml. of solution containing $MgCl_2$ (0·012 M) and $MnCl_2$ (0·004 M); 0·5 ml. substrate neutralized with KOH at the indicated concentration; 0·2 ml. of a salt solution (pH 7·5) consisting of 0·126 M-KCl, 0·0012 M-KH_2PO_4, 0·0106 M-Na_2HPO_4. The substrates used were: casein hydrolysate, 0·3 %; glutamic acid, 0·0125 M;

succinic acid 0·0125 M; pyruvic acid 0·004 M. A control was included in each experiment consisting of 1·5 ml. normal yolk sac suspension in place of psittacosis virus suspension. Manometer readings were taken over a period of 4 hr. No respiratory activity of psittacosis virus could be demonstrated in the presence of any of the four substrates tested.

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FURNESS, G. & GAZE, H. W. (1952). *J. gen. Microbiol.* **6**, 149

A Method of Preparing Individual Sterile Pipettes

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SUMMARY: Tubes of Kraft paper which can be used repeatedly up to six times are used for encasing individual pipettes.

The laboratory practice of wrapping pipettes individually in paper before sterilization has a number of disadvantages. The process is time-consuming, when thin paper is used there is a danger that it may crack and lead to contamination of the pipette, and tough Kraft paper is expensive and scarce. To overcome these difficulties seamless tubes of Kraft paper sufficiently thick to be rigid were obtained from the Star Paper Tube Co. Ltd., Albert Mills, Hollinwood, Oldham. The tubes are 18 in. long, to allow the end to be trimmed, and of $\frac{3}{8}$ in. diameter for pipettes under 5 ml. and $\frac{1}{2}$ in. diameter for those of 5 and 10 ml. capacity; other sizes can be obtained.

Before use one end of the tube is plugged with cotton-wool which is retained in position by stapling with an ordinary office stapler so that this end of the tube is pinched flat with the cotton-wool retained inside. After inserting the pipette the other end of the tube is plugged as for test tubes, different sizes of pipette being identified by differently coloured wools.

The encased pipettes are sterilized in the hot air oven at 160° for 1 hr. The durability of the tubes is such that they can be heated six times at 180° for 1 hr. before they become brittle and crack. They should, however, be inspected each time before use to ensure that they are in a satisfactory condition. The open end of the tube will withstand moderate flaming and can be trimmed if it should become charred.

This method has the following advantages over wrapping. Assuming an average of six sterilizations, the cost of materials is halved, the time required in preparation is about one-quarter and much tedious repetitive work obviated. The cotton-wool and rigidity of the case protect the pipette from accidental damage and allow it to be more easily extracted than from paper wrappers. The procedure has been in routine use in this laboratory for some time and has given general satisfaction.

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SHARPE, M. E. & SHATTOCK, P. M. F. (1952). *J. gen. Microbiol.* 6, 150–165

The Serological Typing of Group D Streptococci associated with Outbreaks of Neonatal Diarrhoea

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SUMMARY: By the preparation of specific type sera 24 types of streptococci within group D are designated on the basis of agglutination tests. Subtypes associated with three of these types are indicated by reciprocal absorption. Precipitin tests with absorbed sera substantiate the division of types by agglutination. Of 353 strains of streptococci identified serologically as group D and further specified by physiological characters, 281 were typed by slide agglutination; the remaining 72 did not fall into any of the 24 types. Whilst there is no absolute correlation between serological type and physiological characteristics, a broad division can be made between *Streptococcus faecalis* and its varieties on the one hand and *Strep. durans*, *Strep. bovis* and various unclassified strains on the other.

The work reported here was carried out in the course of an investigation of the epidemiological significance of group D streptococci in outbreaks of neonatal diarrhoea. Whilst there have been several reports on serological types in this group, there has been little correlation between types designated by various workers. Much of the work has been concerned with the 'enterococci', not always specifically defined, but mainly comprising heat-resistant faecal streptococci. Of the earlier workers, Takeda (1935), together with Shigeno (1937), differentiated 31 types among the 'enterococci', and Meyer (1937) was able to identify six of his serological types with those of Takeda and Shigeno. Grumbach & Schnetz (1938), Grumbach (1943), and recently Skadhauge (1950), have also divided collections of 'enterococci' by agglutination. With streptococci defined serologically as group D a small number of serological types have been reported by Lancefield (1941), Sylvester & Benedict (1941) and Foley & Wheeler (1945).

MATERIALS AND METHODS

Source of cultures

During this investigation group D streptococci were isolated from the faeces of healthy and sick infants, and a collection of 353 representative strains, preserved by freeze-drying, formed the basis for the studies reported here.

Cultural methods

Litmus milk. Reduction, and the formation of acid and/or clot was recorded after incubation at 37° for 24 hr.

Carbohydrate fermentations. Peptone water (5 ml.) containing 0·5 % (w/v) of test sugar added after sterilization, with litmus as indicator, was inoculated with one loopful (4 mm. diameter) of an 18 hr. broth culture and incubated at 37°. Results were read after 7 days.

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Gelatine liquefaction. Stab cultures were incubated at 22° and the results recorded after 10 days.

Haemolysis. Brown's (1919) technique was modified by substituting for the veal infusion basal medium a nutrient agar consisting of agar (2 %), Evans's peptone (1 %), Lemco (1 %) and NaCl (0·5 %).

Growth at 45°. Ten ml. volumes of glucose (1 %) Lemco broth were inoculated with two loopfuls (4 mm.) of an 18 hr. broth culture, incubated in a water-bath at 45° ± 0·1 and examined for growth after 24 hr.

Growth at pH 9·6. The technique described by Shattock & Hirsch (1947) was used.

Tyrosine decarboxylase activity. The technique evolved by Gale (personal communication, see Sharpe, 1948) was followed. It is based on the final pH value reached in a standard medium containing tyrosine, as compared with the pH value reached in the same medium without tyrosine. A difference of 1·2 pH units or more indicates an active organism.

Serological methods

Preparation of immune sera

Group D sera. A strain of *Strep. durans* 98D, used for a number of years in this laboratory for the routine preparation of group D sera, was used throughout. Two methods (Shattock, 1949), both involving disruption of the cells, were used for the preparation of immunizing suspensions.

The first method, in which the organisms were extracted with acetone before being ground in a ball mill, was later replaced by the more satisfactory method of disrupting the organism in a vibrating machine (Mickle, 1948), with carborundum powder grade 100.

Type sera. To the centrifuged cells from 500 ml. of an 18 hr. glucose (1 %) Lemco broth culture, 20 ml. of 0·85 % NaCl containing 0·25 % formalin were added. The suspension was left at room temperature for a few days, tested for sterility, and then stored in the cold. For inoculating into rabbits, suspensions equivalent to Brown's opacity tube no. 7 (Burroughs Wellcome and Co. Ltd.) were prepared. A course of five intravenous inoculations (0·25 ml., 0·5 ml. and three of 1·0 ml.) given at 3- or 4-day intervals usually resulted in type sera of satisfactory titre. Titres varied from 640 to 10,240; with two strains a titre of only 320 could be reached. In the few instances where a low titre was obtained after the first course it was found difficult to increase.

Group and type sera were stored in the cold without preservative.

Precipitin tests

Preparation of HCl *extracts.* HCl extracts were prepared by Lancefield's (1933) method, extracting the centrifuged organisms from 50 ml. of 24 hr. glucose (1 %) Lemco broth cultures with 1·5–2·0 ml. 0·05 N-HCl in saline. Extracts which did not react with a group D serum were precipitated by ethanol (fraction A, Shattock, 1949) to concentrate any group D substance present.

Preparation of formamide extracts. Using a modification of Fuller's (1938) method, the deposits from 10 ml. of 18 hr. glucose (1 %) Lemco broth cultures were extracted with 1·0 ml. formamide at 150° for 15 min., purified by adding 2·5 ml. acid ethanol (95 parts ethanol, 5 parts HCl, 20 %, v/v) and discarding the precipitate. Acetone (5·0 ml.) was added to the supernatant and the resulting precipitate resuspended in 1·0 ml. saline.

Precipitin test. Extract was layered on serum in a small glass tube of internal diameter 3·0 mm. and examined for ring formation at room temperature against a standard diffused light, at intervals up to 30 min.

Precipitin absorption. Type sera showing cross-reactions by the precipitin ring test were absorbed to remove non-specific antibodies. Two volumes of undiluted serum were absorbed with 1 vol. of packed streptococci previously washed and killed by heating to 59° for 1 hr. Absorption was carried out at 37° for 2 hr. followed by overnight refrigeration.

Agglutination tests

Slide agglutination tests. A slight modification of Stableforth's (1932) method was followed. Cultures were grown in 10 ml. of glucose (0·2 %) Lemco broth for 6 hr. at 37°, centrifuged and resuspended in 0·3 ml. saline containing 0·25 % formalin. Tests were usually made with absorbed type specific sera, final dilutions being either 1/32, 1/64 or occasionally 1/128 of the titre. Spontaneous agglutination occurred occasionally, but this could usually be avoided by decreasing the time of incubation of the culture from 6 to 4 hr.

Tube agglutination tests. Bacterial suspensions for agglutination were prepared from 6 hr. glucose (0·1 %) Lemco broth cultures incubated at 37° preserved with 0·25 % formalin and stored in the cold. No difficulty with rough suspensions was encountered. Dreyer's agglutination technique was followed. Results were read after overnight incubation at 50°.

Agglutinin absorption. For absorbing suspensions the organism was grown in 500 ml. of glucose (0·2 %) Lemco broth for 48 hr. at 37°, the centrifuged organisms resuspended in 0·25 % formalin saline to give a concentration of $20,000 \times 10^6$ cocci/ml. and stored in the ice chest. Colebrook (1935) noted no difference between formolized and unformolized suspensions of group A streptococci used for absorption, and formolized suspensions were therefore used throughout this work.

Sera were diluted to 1/64 of their titres, absorbed with an equal volume of absorbing suspension at 37° and the degree of absorption determined by a 'five-tube' titration as described by Colebrook (1935). A diminution of titre by 1, 2, 3, 4 or 5 tubes showed a 50, 75, 87·5, 93·75 and 96·8 % decrease of agglutinins respectively. Absorptions resulting in a decrease of 96·8 % or, in some cases, 93·75 % of the agglutinins were taken as indicating complete absorption; a decrease of one tube (50 %) was regarded as insignificant in accordance with Colebrook's opinion.

The minimum absorbing dose was determined by absorbing a small quantity of the diluted serum with an equal volume of homologous suspension containing $20,000 \times 10^6$ cocci/ml. for 2 hr. at 37° and titrating the absorbed serum for agglutinins. When agglutinins were still present, further absorptions were carried out, and the initial dilution of serum adjusted to give a final dilution of 1/32 of the titre. In some cases sera had to be absorbed three times with the homologous organism before complete absorption was attained, but usually two such absorptions were sufficient. The minimum absorbing dose for a serum having been determined, all absorptions with heterologous organisms were carried out in a similar manner.

RESULTS

Serological grouping. All 353 strains of streptococci examined were shown by precipitin tests to be group D.

Serological typing

Choice of strains for the preparation of type sera. Much of the serological typing reported here was done concurrently with the isolation and identification of fresh cultures, so that new types were found during the course of the work. A preliminary physiological division, such as might have been done with a complete collection of identified organisms, was therefore not possible. Six type sera were first prepared from strains isolated from faeces of healthy infants, each strain having different physiological characteristics; later, however, strains for the production of type sera were chosen for their possible epidemiological significance.

Type specific sera. Twenty-four specific types were separated using tube agglutination and absorption of agglutinins, with reciprocal absorption tests when necessary, and it was evident that other types also existed.

Absorption of non-specific agglutinins. Tube agglutination tests against heterologous suspensions showed that many cross-reactions occurred with most of the sera. In every case where the titre with the heterologous suspension was as high as, or one dilution lower than, that with the homologous suspension, reciprocal absorption tests were done to determine whether or not the two strains were identical. It was found that most of these cross-reactions were non-specific and could be eliminated by absorption with appropriate suspensions. For absorption a heterologous strain reacting to a high titre with the serum was used, at the minimum absorbing dose determined with the homologous organism. The dose varied with each strain and was not associated with the titre of the serum. This was also noted by Torrey & Montu (1934) in their work on streptococci from ulcerative colitis. A number of trial tests using small amounts of sera were necessary before absorbing a serum in quantity, as the selection of strains for the removal of non-specific agglutinins sometimes proved to be a matter of trial and error. With some sera it was necessary to absorb with strains of two different serological types, neither alone being capable of absorbing all the non-specific antibodies.

Many of the sera, particularly those for *Strep. bovis*, were easier to absorb, showing only a few low titre non-specific cross-reactions which were removed by absorbing once with a heterologous type. Other sera presented slightly greater difficulties, but suitable absorbing strains were found for all type sera, and specific sera for slide agglutination were prepared for all 24 types. Strain GB 122 (type 4) was particularly useful for absorbing non-specific antibodies.

Absorption with minimum absorbing doses of heterologous organisms rarely lowered the titre of the serum to the homologous strain, even by one tube in the 'five-tube' test; the few strains which did cause such a drop in titre were not used for specific absorptions.

Nature of non-specific reactions. The non-specific reactions did not appear to be due to group specific antibodies. Attempts were made to absorb two sera with *Strep. durans* 98D, the strain from which group D serum was prepared, and therefore, presumably, rich in group substance. There was, however, little absorption of non-specific agglutinins. Indeed there is little likelihood of frequent cross-reactions due to group specific antibodies in type sera produced by comparatively few inoculations of whole organisms, as group D sera are notoriously troublesome to prepare.

Agglutination tests with specific absorbed sera. A complete series of reciprocal slide agglutination tests showed that 22 of the 24 type sera gave no cross-reactions (Table 1). Two sera, types 5 and 9, gave slight reciprocal cross-reactions but were specific towards the remaining 22 type suspensions.

Reciprocal absorption tests with types 5 and 9 were carried out. A suspension of type 5 did not absorb the agglutinins of serum type 9 even to the extent of lowering the titre by one tube in the 'five-tube' test. However, the suspension of type 9 absorbed 87·5 % of the agglutinins of serum type 5 so that some antigenic relationship is indicated. Perhaps the major type antigen in 5 is a minor one in 9, or the two type antigens may be similar enough in constitution for some absorption of one by the other to take place. In practice the slow incomplete cross-reactions in slide agglutination tests with these two sera were readily differentiated from the rapid complete agglutination of the organisms with their homologous sera. This was not the case with subtypes referred to later.

Precipitin tests. Precipitin tests with the 24 undiluted type sera were made with both HCl and formamide extracts of all types. Using unabsorbed sera, HCl and formamide extracts gave strong reactions with their homologous sera in every case, but frequent non-specific cross-reactions were also observed.

Type sera exhibiting non-specific reactions were absorbed with strain GB122 (type 4); tests with HCl extracts on absorbed sera showed that most of the cross-reactions had been eliminated. Results with precipitin tests confirmed the relationship found with agglutination tests between type 5 and type 9, but, in addition, sera for both types 5 and 9 reacted with an extract of type 23; cross-reactions also occurred with type 18 serum and an extract of type 11.

Typing of strains from the faeces of infants. Table 2 shows the serological typing of 353 strains of group D streptococci by the slide agglutination technique. Type 1 predominated, whilst five types were found only once. The epidemiological significance of these types will be dealt with in another paper. Seventy-two strains did not fall into any of the 24 types for which specific sera had been prepared, but as no technical difficulties with suspensions were

Table 1. *Cross agglutination (slide) tests with absorbed type sera and suspensions of type organisms*

Type suspensions	Type sera																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	+	−	−	−	±*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	±*	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
13	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
21	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
24	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

* ± denotes a slow reaction.

encountered it is probable that further specific sera could be prepared and all strains eventually typed serologically. Whilst only one serum was prepared from each of the more uncommon types, sera were made from several strains of the more prevalent types, and their identity checked by reciprocal absorption tests.

Table 2. *Distribution of* 353 *strains of group D streptococci among the* 24 *serological types; by slide agglutination*

Serological type	No. of strains of each type	Serological type	No. of strains of each type
1	83	14	8
2	7	15	1
3	11	16	8
4	7	17	12
5	28	18	4
6	24	19	7
7	8	20	1
8	23	21	1
9	33	22	2
10	2	23	1
11	2	24	1
12	3	Untyped	72
13	4		

Subtypes. With some strains reciprocal absorption showed the existence of closely related but not identical types; these were regarded as subtypes. They were not differentiated by slide agglutination, but were encountered during random samplings of strains of the three more common serological types for the confirmation of slide agglutinations by the more delicate tube technique. This is in contrast to the relationship between types 5 and 9 which were readily differentiated by slide agglutination. These data are presented in Table 3. In type 1, strain B17 completely absorbed serum N37, but strain N37 did not absorb serum B17. In the same way, in type 5, strain GB141 entirely removed the agglutinins from serum N83, but N83 did not do so for serum GB141, whilst in type 9 strain GN23 completely absorbed N97, but strain N97 did not absorb GN23 completely. These findings suggest that strains B17, GB141 and GN23 were closely related to types 1, 5 and 9 respectively, and should be regarded as subtypes. The subtype may possess an extra antigen. No attempt was made to find more of such related types, and it was not determined how many of the strains originally classified as types 1, 5 and 9 would fall into the subtypes.

Attempts to differentiate subtypes by precipitin tests were unsuccessful. Using unabsorbed sera, extracts and sera of type and subtype reacted equally well with each other. Reciprocal absorption tests proved inconclusive. In several instances the absorbing organism did not absorb all the homologous antibodies, and attempts at further absorption resulted in complete absorption of sera, so that no differentiation of subtype could be made.

Table 3. *Reciprocal absorption tests indicating the presence of subtypes*

Serological type	Strain used for preparation of serum	Absorbing strain	Agglutination titre against homologous strain
1	N37	Unabsorbed	10,240
	N37	N37	<640 (96·8 %)[*]
	N37	B17	<640 (96·8 %)
	B17	Unabsorbed	2,560
	B17	B17	<160 (96·8 %)
	B17	N37	2,560[†]
5	N83	Unabsorbed	2,560
	N83	N83	<160 (96·8 %)
	N83	GB141	<160 (96·8 %)
	GB141	Unabsorbed	1,280
	GB141	GB141	<80 (96·8 %)
	GB141	N83	640[†] (50 %)
9	N97	Unabsorbed	5,120
	N97	N97	<320 (96·8 %)
	N97	GN23	<320 (96·8 %)
	GN23	Unabsorbed	2,560
	GN23	GN23	<160 (96·8 %)
	GN23	N97	2,560

[*] Figures in brackets indicate degree of absorption.

[†] Although the titre was little altered, the amount of agglutination present in the tubes was very much reduced.

Physiological characters of serological types

In addition to the usual physiological tests for the differentiation of species and varieties within group D (Shattock, 1949), the presence of tyrosine decarboxylase was examined. Sharpe (1948) found that the production of this enzyme was a useful differential characteristic in addition to those usually accepted in the subdivision of group D.

Table 4 gives the physiological reactions of the 24 strains upon which the serological typing is based, together with those of other strains found by reciprocal absorption to be identical with the type strains; the physiological reactions of subtypes are also given. The results concerning the survival at 60° for 30 min., a test often applied in the differentiation of group D, are omitted from Table 4. The unreliability of this test was again demonstrated in this work, confirming the opinion of Shattock & Mattick (1943). All species and varieties of group D are included and it will be seen that physiological divisions within group D are substantiated by these serological studies. *Strep. faecalis* and its varieties *liquefaciens* and *zymogenes* are associated serologically in types 1, 3, 5 and 9, whilst *Strep. durans* and *Strep. bovis* have their own respective serological types.

These points are further illustrated in Table 5, which summarizes the physiological activities of 353 strains of group D streptococci, together with the serological typing of 281 of these as classified by slide agglutination into the 24 designated types. As no attempt was made to define any subtypes which might be present, main and subtypes are included as the same type.

Table 4. *Physiological characters of the type strains of group D streptococci and of their subtypes*

Serological type	Culture strain	Litmus milk		Mannitol	Sucrose	Raffinose	Liq. gelatin	Growth at		Haemo-lysis	Tyrosine decarbox. activity, pH difference*	Species identification
		1 day	7 days					45°	pH 9·6			
1	N37	RA	RAC	A	A	–	–	+	+	β	1·56	var. *zymogenes*†
	S12	RA	RAC	A	A	–	–	+	+	Greening	1·49	*Strep. faecalis*
	S65	RA	RAC	A	A	–	–	+	+	Greening	1·72	*Strep. faecalis*
	S230	RACD		A	A	–	+	+	+	Greening	1·57	var. *liquefaciens*†
1 subtype	GB112	RA	RAC	A	A	–	–	+	+	β	1·63	var. *zymogenes*
2	S182	A (sl.)	ACR	–	A	A	–	+	–	α	0·02	*Strep. bovis*
3	S161	RAC	RAC	A	A (sl.)	–	–	+	+	Greening	1·55	*Strep. faecalis*
	D41	RA	RAC	A	A (sl.)	–	–	+	+	β	1·29	var. *zymogenes*
	Nu13	RAC	RAC	A	A	–	–	+	+	Bleaching	1·50	*Strep. faecalis*
4	GB122	RACD		A	A	–	+	+	+	Greening	1·36	var. *liquefaciens*
5	N83	RA	RAC	A	A	–	–	+	+	Greening	1·70	*Strep. faecalis*
	Nu17	RAC	RACD	A	A	–	+	+	+	α	1·52	var. *liquefaciens*
5 subtype	GB141	RA	RAC	A	A	–	–	+	+	β	1·53	var. *zymogenes*
6	B65	RA	RAC	A	–	–	–	+	+	Greening	1·47	*Strep. faecalis*
	B47	RA	RAC	A	–	–	–	+	+	Greening	1·55	*Strep. faecalis*
7	B26	A (sl.)	AC	A	A	–	–	+	–	α	0·00	Unclassified
8	B8	RA	RAC	A	A	–	–	+	+	Greening	1·49	*Strep. faecalis*
	IAS	RA	RAC	A	A	–	–	+	+	Greening	1·57	*Strep. faecalis*
9	N97	RAC	RACD	A	A	–	+	+	+	Greening	1·29	var. *liquefaciens*
	D1	RA	RAC	A	A	–	–	+	+	β	1·57	var. *zymogenes*
9 subtype	GN23	rA	RAC	A	A	–	–	+	+	Greening	1·41	*Strep. faecalis*
10	N42	RAC	RAC	A	A	A	–	+	+	Greening	0·04	Unclassified
11	N16	A (sl.)	AC	A (sl.)	–	–	–	+	+	α	1·06	*Strep. durans*
12	S194	A (sl.)	AC	–	A	A	–	+	–	α	0·16	*Strep. bovis*
13	S112	A	AC	–	A	A	–	+	–	α	0·08	*Strep. bovis*
	S71	–	AC	–	A	A	–	+	–	α	0·01	*Strep. bovis*
14	N55	A (sl.)	AC	A	–	–	–	+	+	α	0·93	*Strep. faecalis*
15	B74	RAC	RAC	A	A	A	–	+	+	α	0·02	Unclassified
16	N205	A (sl.)	AC	A	A	–	–	+	+	Greening	0·00	*Strep. faecalis*
17	L16	A	ACR	–	A	–	–	+	–	Greening	0·03	Unclassified
18	S98	RAC	RAC	A	–	–	–	+	+	α	1·42	*Strep. faecalis*
19	D36	RAC	RACD	A	A	–	+	+	+	Greening	1·45	var. *liquefaciens*
20	N161	RAC	RACD	A	A	–	+	+	+	Greening	1·46	var. *liquefaciens*
21	L6	A	ACR	–	A	–	–	+	–	Bleaching	0·03	Unclassified
22	H2	A (sl.)	ACR	–	–	–	–	+	+	Bleaching	1·12	*Strep. durans*
23	H6	A (sl.)	A (sl.)	A	A	A	–	+	–	α	0·14	*Strep. bovis*
24	H24	A (sl.)	ACR	A	A	–	–	+	+	Greening	0·86	*Strep. faecalis*

A = acid; A (sl.) = slight acid; R = complete reduction; r = partial reduction; C = clot; D = digestion.

* Tyrosine decarboxylase activity measured as difference between the pH value reached in a standard medium containing tyrosine and the value reached in the same medium without tyrosine.

† Var. *zymogenes* indicates *Strep. faecalis* var. *zymogenes*, var. *liquefaciens* indicates *Strep. faecalis* var. *liquefaciens*.

Table 5. *Association of serological type with physiological characters of group D*

Species of group D	Physiological characters									Number of strains of each serological type falling into each physiological category																										
										Section I												Section II														
										Strep. faecalis group Type												*Strep. durans* Type		*Strep. bovis* Type				Unclassified Type								
	Litmus milk 24 hr.	Mannitol	Sucrose	Raffinose	Gelatin	Growth at pH 9·6	Growth at 45°	Tyrosine de-carboxylase activity	Haemolysis	1	3	4	5	6	8	9	14	18	19	20	24	11	22	2	12	13	23	7	10	15	16	17	21	Unclassified*	Total number of strains
Section I:																																			
Strep. faecalis	RAC	A	A±	−	−	+	+	+	α	59	6	.	18	23	21	18	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	12	158
Strep. faecalis var.	A	A	A±	−	−	+	+	+	α	.	.	.	2	.	.	.	7	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	19	31
Strep. faecalis var. *liquefaciens*	RAC	A	A	−	+	+	+	+	α	13	1	7	2	1	2	12	.	.	7	1	.	.	.	.	.	.	.	.	.	.	.	.	.	7	53
Strep. faecalis var. *zymogenes*	RAC	A	A	−	±	+	+	+	β	11	4	.	6	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	25
Section II:																																			
Strep. durans	A	−	−	−	−	+	+	±	α	.	.	.	.	.	.	.	.	.	.	.	.	1	2	.	.	.	.	.	.	.	1	.	.	3	7
Strep. bovis	A	A±	A	A	−	−	+	−	α	.	.	.	.	.	.	.	1	.	.	.	.	.	.	7	3	3	1	4	.	.	.	1	.	11	31
Unclassified	A	−	A	−	−	+	+	+	α	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1
Unclassified	A	−	A	−	−	−	+	+	α	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	1	.	.	.	2
Unclassified	A	−	A	−	−	−	+	−	α	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	11	1	5	17
Unclassified	A	A	A±	−	−	+	+	−	α	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	4	.	.	4	9
Unclassified	A	A	A±	−	−	−	+	−	α	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	4	.	.	1	.	.	4	10
Unclassified	RAC	A	A	A	−	−	+	−	α	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	.	.	.	6	9
Total number of strains in each type										83	11	7	28	24	23	33	8	4	7	1	1	2	2	7	3	4	1	8	2	1	8	12	1	72	353

* Did not fall into any of the 24 types for which specific sera were available.
+ indicates a tyrosine decarboxylase activity of over 0·6 pH unit.
− indicates a tyrosine decarboxylase activity of less than 0·6 pH unit.
α includes 'greening' or 'bleaching' as well as true α haemolysis.
A ± = some strains producing acid and others not producing acid.

It will be seen that strains differing from *Strep. faecalis* only in failing to reduce litmus milk in 24 hr. have been included as *Strep. faecalis.* Strains differing from *Strep. faecalis* in that they fermented raffinose and had little or no tyrosine decarboxylase are included as unclassified strains (Shattock, 1949), and have been grouped with *Strep. bovis* rather than with *Strep. faecalis.* Of nine such strains three were typed, two being type 10 and one type 15; as no strains with the physiological reactions of *Strep. faecalis* or *Strep. bovis* fell into either of these types, no serological information was available for deciding whether these variants were more likely to be allied to *Strep. faecalis* or to *Strep. bovis.*

Whilst there was no absolute correlation between physiological reactions and serological type, it was possible to divide the type strains into two sections in which physiological and serological types were broadly associated (Table 5). In one section (I) the type sera were made from organisms of typical *Strep. faecalis* and varieties, in the other section (II) sera were prepared from *Strep. bovis, Strep. durans* and unclassified strains. Type sera in section I agglutinated only organisms with the physiological reactions of section I with two exceptions, namely sera for types 14 and 18 (section I) each agglutinated one strain falling into section II. Type sera in section II agglutinated only organisms having the physiological reactions of this section. It can be seen that types 1, 3, 5 and 9 contained all three varieties of *Strep. faecalis.* A variant which did not rapidly reduce litmus milk also fell into type 5. Types 6 and 8 contained *Strep. faecalis* and *Strep. faecalis* var. *liquefaciens*, type 18 contained *Strep. faecalis* and a non-reducing variant, whilst strains of only one species were found in types 4, 19, 20 and 24. With the examination of strains from other sources it is possible that all serological types might be shown to contain more than one physiological variety.

In section II strains with a wider variety of biochemical reactions occur. Type 16 contains tyrosine decarboxylase positive and negative strains, and strains which ferment or fail to ferment mannitol. Into types 7, 17 and 13 fall raffinose-fermenting and non-fermenting strains. However, *Strep. bovis* sera showed greater specificity, only one strain not giving the reactions of *Strep. bovis* was agglutinated by a *Strep. bovis* serum (type 13). Seventy-two strains of many different physiological reactions remained untyped.

Comparisons with group D serological types of other workers

Lancefield (1941) mentioned that she had identified three serological types of group D, and Foley & Wheeler (1945) used these three together with a fourth type for work on serological and physiological characteristics of group D. Dr Lancefield kindly sent to this laboratory two of her type strains (H69D5, D76), which were also used by Foley & Wheeler. They were examined, by slide agglutination only, and identified as falling into the Reading types. H69D5 (Foley & Wheeler type 1) reacted with our type 3 serum, and D76 (Foley & Wheeler type 4) with our type 1 serum. H69D5 was the most prevalent type in Foley & Wheeler's investigations, 100 out of 136 strains falling into this type, whilst D76 was uncommon, only two strains being found. In the work reported here, of 353 strains 83 were type 1 and 11 strains were type 3. The physiological reactions reported by Foley & Wheeler, in so far as they could be compared, were similar to those detailed here.

Mention has been made of Grumbach's (1943) 21 serological types, into which he classified 468 out of 597 strains of 'enterococci'. Prof. Grumbach was kind enough to send 14 of his type sera which were available, and also his 21 type strains. Unfortunately it was not possible to make any satisfactory comparisons as many of his strains and sera had deteriorated, possibly owing to their age as apparently sera and cultures had been stored for several years. It has been our experience that type sera tend to lose activity after approximately 3 years storage in the ice chest.

Since this work was completed, Skadhauge (1950) has published his monograph, *Studies on Enterococci, with Special Reference to the Serological Properties*. His strains were selected on the basis of physiological reactions and classified as *Strep. faecalis* and its varieties (*liquefaciens, zymogenes, haemolyticus*), as *Strep. durans*, or as 'unclassified'. By testing the capacity of all strains to grow in a medium containing potassium tellurite (1/2500) they could be divided into tellurite resistant and tellurite sensitive strains. In the resistant group occurred *Strep. faecalis* and its varieties, forming a culturally uniform group, all of which fermented sorbitol. In the tellurite sensitive group occurred strains of more heterogeneous characteristics, including *Strep. durans*, various unclassified strains, and some strains of *Strep. faecalis* which fermented arabinose but not sorbitol. By this classification the group of *Strep. faecalis* and its varieties was more closely defined than previously. The tellurite sensitive strains of *Strep. faecalis* showed a striking cultural resemblance to *Strep. faecium* of Orla-Jensen (1919), and a comparison with Orla-Jensen's original cultures of *Strep. faecium* showed that they also were tellurite sensitive. This test also differentiated *Strep. faecium* from Orla-Jensen's *Strep. glycerinaceus*, which fell into the *faecalis* group and was tellurite resistant. Skadhauge suggested that these results confirm Orla-Jensen's classification of *Strep. faecium* as a separate species, and that it should not be regarded as synonymous with *Strep. faecalis*. In serological studies, using agglutination and agglutinin-absorption tests, Skadhauge demonstrated two somatic antigens, one thermostable (O) and one thermolabile (K), and with some strains a capsular heat-stable antigen (A). *Strep. faecalis* and its varieties could be divided into at least four groups based on the O antigens. In contrast, the various *Strep. faecium, Strep. durans* and unclassified strains examined showed no O antigen relationships on which grouping could be based. The capsular antigen A, demonstrated only in some strains of *Strep. faecium*, inhibited O agglutination. The K antigen was assumed to be localized on the surface of the cells and gave a wider range of activity than the O antigen. None of the antigenic relationships, however, were as extensive as the Lancefield group D antigen.

These findings are of considerable interest in relation to the work reported here. Whilst no comparison between the Reading serological types and Skadhauge's O groups has yet been possible, 23 of the 24 Reading type strains have been tested for resistance to potassium tellurite (Table 6). Cultures

(18 hr.) were plated on the surface of an agar medium consisting of yeast glucose agar + 10 % (v/v) horse blood, + potassium tellurite 1/2500 heated to 70° for 10 min. The ability of strains to ferment sorbitol and arabinose was also examined. The results did not entirely agree with Skadhauge's findings, as four of the tellurite-sensitive strains fermented sorbitol.

Table 6. *Further cultural reactions of the Reading type strains*

Serological type	Resistance to tellurite	Fermentation of	
		Sorbitol	Arabinose
1	+	A	–
3	+	A	–
4	+	A	–
5	+	A	–
6	+	A	–
8	+	A	–
9	+	A	–
19	+	A	–
20	+	A	–
2	–	–	–
7	–	A (slow)	A
10	–	–	A
11	–	–	A
12	–	–	–
13	–	–	–
14	–	–	A
15	–	A (slight)	A
16	–	–	A
17		Not tested	
18	–	A	A
21	–	–	–
22	–	–	A
23	–	A	A
24	–	–	A

A comparison of this result with those given in Table 5 shows similarity in grouping. Section I, the *faecalis* group, contains all the tellurite resistant strains, but, in addition, types 18, 14 and 24 which are tellurite sensitive. It is interesting that two of these types, 14 and 18, each contain one strain which did not fall into section I, and that 14, 24 and 18, with the exception of one strain, differ from the typical varieties of *Strep. faecalis* in that they do not reduce litmus milk rapidly. If these three types which conform to Skadhauge's description of *Strep. faecium* were removed from section I to section II (Table 5), the former is even more closely defined. However, an investigation of the tellurite resistance of a large number of strains of each serological type is necessary before further conclusions can be drawn.

DISCUSSION

Our demonstration of many serological types among group D streptococci agrees with the findings of Takeda (1935) and Shigeno (1937) whose combined agglutination typing of 'enterococci' from the human intestine yielded 31 serological types, and Grumbach (1943) who obtained 21 types all of which gave typical physiological reactions of *Strep. faecalis* or one of its varieties.

Subtypes, as found here for group D, have also been found in group B (e.g. Stableforth, 1937; Stewart, 1937; Simmons & Keogh, 1940). The subtypes in group B differ from those in group D, however, in that specific sera for each B subtype could be obtained by absorption with any related subtype. These B subtypes then, besides possessing an antigen in common, each possessed a specific one which gave strong enough agglutination reactions to distinguish between them. With group D, however, the relationship is slightly different. When the main types are absorbed with the subtypes, complete absorption (96·8 %) of the antibodies occurs. When the subtypes are absorbed with main types, complete absorption does not occur. Hence the subtype must possess an extra antigen.

The agreement between typing by agglutination and by precipitation noted by Foley & Wheeler (1945) for four types within group D has also been mainly our experience, but whether identical antigens are responsible for results by both precipitation and agglutination has not been determined. In the serological typing of group A, Pauli & Coburn (1937) found that absorption of crude sera with the purified group specific carbohydrate gave type specific agglutinating sera, but Rudd, White & Ward (1939), were unable to absorb more than 50 % of the non-type specific antibodies in this way, and considered it doubtful whether group antibody is responsible for all cross-reactions. In our experience with group D the fact that absorption with different types is necessary, and that more than one type is sometimes required, seems to indicate that several non-specific antigens are involved in agglutination tests.

The lack of association between serological type and certain physiological characteristics observed here has also been recorded by other workers. Torrey & Montu (1934) found that mannitol-positive, raffinose-negative strains fell into the same types as mannitol-negative, raffinose-positive strains by agglutination test. Heat resistance and lack of it was exhibited by identical types, and *Strep. zymogenes* strains were very closely related to a mannitol-negative, raffinose-positive *β* haemolytic strain. Although absorbed sera were used it is possible that the latter relationship was due to non-specific reaction; in our experience *Strep. faecalis* var. *zymogenes* and raffinose fermenting organisms were never encountered in the same serological type.

Foley & Wheeler (1945) found that neither heat resistance, inability to ferment mannitol nor haemolytic activity were related to serological type, and Grumbach (1947) recorded, as indeed have we, that several serological types had identical cultural characteristics.

It is generally agreed, then, that serological typing is not closely correlated with the physiological tests in general use. The work presented here does indicate, however, that serological types fall into two broad sections which appear to be associated with general physiological reactions, one including *Strep. faecalis* and its varieties, and the other *Strep. bovis*, *Strep. durans* and unclassified strains. Further work on a larger number of strains from a wider variety of sources is necessary to confirm or disprove this, or even to make further divisions possible.

Some further consideration must be given here to Skadhauge's (1950) work on the 'enterococci'. It has already been mentioned that none of the antigenic relationships elaborated by him were comparable with the Lancefield group D antigen. It is unfortunate that he was unable to prepare a group D serum for this purpose, as serological studies based on the Lancefield grouping have done much to elucidate relationships among the faecal streptococci and other members of the genus. However, he made precipitin tests on a limited number of extracts of strains, classified on their cultural properties, with group D sera prepared by Lancefield and by Shattock. With the latter's serum, obtained with organisms extracted with acetone and ground in a ball-mill (Shattock & Mattick, 1943), all strains specified as *Strep. faecalis* and *Strep. faecium* reacted, whilst with Lancefield's serum several strains of *Strep. faecalis* and *Strep. durans* and all strains of *Strep. faecium* failed to react. This discrepancy may well have been due to deterioration of the group antibody during storage, which does occur with some batches of group D serum. We agree that a *prima facie* case has been made for the separation of tellurite sensitive strains corresponding to the *Strep. faecium* of Orla-Jensen, and if further serological work confirms the preliminary findings reported both by Skadhauge and by us, it seems that group D should be further divided to include *Strep. faecium* as a separate species.

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These type strains, with the exception of type 17 which has been lost, have been deposited with the National Collection of Type Cultures.

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The Determination of the Most Probable Numbers of Streptomycin-fast Cells in Brucella Cultures and their Variability in Growing and Ageing Cultures

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SUMMARY: The method of most probable numbers (MPN) was employed in order to determine the number of resistant brucella cells at concentrations of streptomycin of 1, 10 and 100μg./ml. The examination showed agreement with the centrifugation method (plating of the sediment of at least 100 ml. of culture together with the required concentration of streptomycin) in a range up to 1000 resistant cells/100 ml. The method showed that there exist differences in the numbers of resistant cells in different strains, and that in cultures originating from the same colony variations occur. The general trend is a relative increase of the resistant cells with the age of the culture.

Methods for determining the number of streptomycin-resistant cells in bacterial cultures have been described by many authors. Klein & Kimmelman (1946) employed broth cultures of *Shigella dysenteriae*. Tenfold dilutions up to 10^{-6} were assayed in varying concentrations of streptomycin in melted agar, the test requiring at least 42 plates. In another experiment they distributed 40 ml. of broth culture into 400 broth tubes containing 1000 units streptomycin/ml. After 48 hr., growth appeared in 5 out of 400 tubes. Yegian & Vanderlinde (1948) incubated different sizes of inocula of *Mycobacterium ranae* on glycerol agar with varying quantities of streptomycin; one test required at least 120 plates. English & McCoy (1951) incubated 100 tubes containing 0·3 ml. broth culture of *Micrococcus pyogenes* and 200 units of streptomycin. One tube out of 100 showed visible growth after 24 hr. They estimated the number of bacteria incubated in 100 tubes at 67×10^8 and concluded, therefore, that one resistant cell was present in this total bacterial population. All these methods require large quantities of tubes, agar and plates, and are time and material consuming. We tried, therefore, to simplify this technique by the determination of the most probable number (MPN) of streptomycin resistant micro-organisms in a culture. This method was proposed by McCrady (1915) for the numerical interpretation of fermentation tube results. For our purposes, the determination of small numbers of streptomycin resistant cells in a large bacterial population, we used the tables published by Hoskins (1934), reprinted in *Standard Methods for the Examination of Water and Sewage* (1936). We expected that with this relatively simple technique it would be possible to detect the presence of streptomycin resistant organisms in brucella cultures at different stages of their incubation.

METHODS AND RESULTS

Preliminary experiments were made to determine the action of streptomycin on the whole bacterial population, and the time of the first visible growth of resistant bacteria at different levels of streptomycin. The methods employed were as follows:

Media. The liquid media contained, per litre, 30·0 g. dehydrated trypticase soy broth (Baltimore Biological Laboratories), 30·0 ml. glycerol and 1·0 ml. of a solution of aneurin containing 0·1 mg./ml.

Strains. Two strains of *Brucella melitensis*, M5 and M7, two strains of *Br. suis*, S39 and S6, and two strains of *Br. abortus*, A19 and A2308, were used. After several subcultures on the above medium 24 hr. cultures were used for the experiments described below.

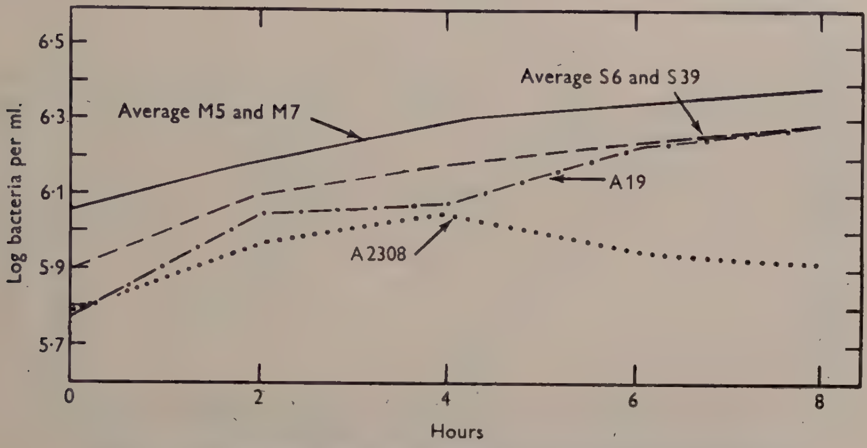

Fig. 1. The changes in bacterial count within 8 hr. in broth containing 1·0 μg. streptomycin/ml.

Streptomycin. Streptomycin sulphate (Squibb) was used in all experiments.

Technique. All experiments were carried out in test-tubes of 20 mm. diameter. The total volume of liquid medium containing the streptomycin and the bacterial inoculum was 10·0 ml. The wide test-tubes containing this relatively small quantity of fluid were thoroughly agitated and then incubated at 37°. Small samples of tube contents were taken at intervals, serial dilutions prepared (1/40, 1/1600, 1/64,000, 1/2,560,000) and 1·0 ml. of each dilution poured to agar plates. At those stages of the experiments where the bacterial count was low the samples were diluted less, and finally the undiluted contents of the tube were poured into agar plates. Fig. 1 represents the number of micro-organisms/ml. found at different intervals within 8 hr. in contact with 1·0 μg. streptomycin/ml. Of the six strains examined five tended to grow, while one strain (A2308) after an initial rise showed a definite decrease in the number of organisms, beginning at the fourth hour.

Fig. 2 shows the behaviour of the bacteria from the tenth hour after contact with 1·0 μg. streptomycin/ml. All strains show a gradual decrease of the number of bacteria; two (M7 and A2308) were completely sterile after 24 hr.; the other four strains contained many living bacteria.

Fig. 3 shows the further behaviour of the four surviving strains. All of them showed on the following days in contact with streptomycin a steady increase of their bacterial count, strain M5 after a slight decrease continuing 2 days. This behaviour of the micro-organisms in the presence of streptomycin was modified under different conditions. With a greater inoculum the number of bacteria did not decrease below 10^6/ml. and a S-shape curve resulted. When the concentration of streptomycin was 100 μg./ml. the number of bacteria decreased after 4 hr. incubation.

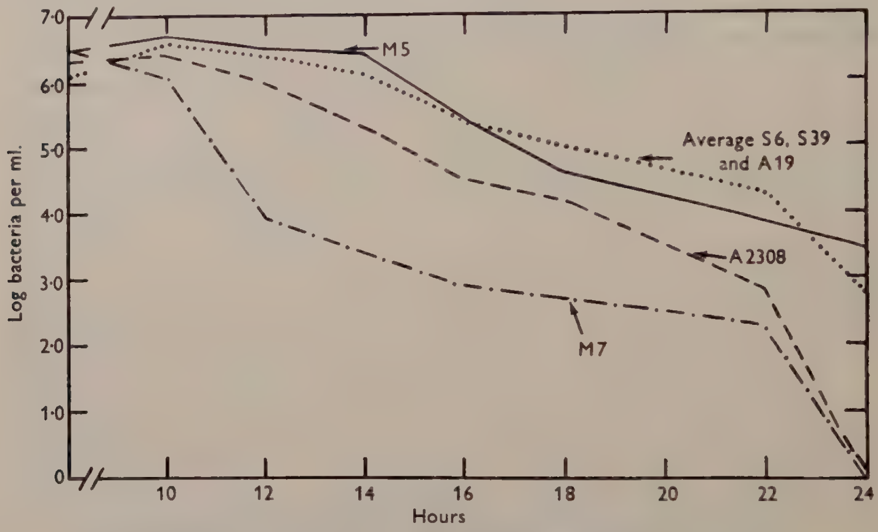

Fig. 2. The changes in bacterial count within 24 hr. in broth containing 1·0 μg. streptomycin/ml.

These experiments showed that the rate of disappearance of the non-resistant bacteria and the time of the initial growth of the resistant bacteria depend upon the concentration of streptomycin and upon the number of organisms in the inoculum. It was necessary to decide whether the bacteria resistant to a given concentration of streptomycin were resistant only to this concentration or whether they had acquired complete resistance. In an experiment carried out with three different inocula of strain M5 exposed to 1·0 μg. streptomycin/ml. for 24 hr. the following results were obtained:

Initial inocula/10 ml.	$6{\cdot}5 \times 10^6$	$1{\cdot}3 \times 10^7$	$2{\cdot}6 \times 10^7$
Surviving bacteria after 24 hr./10 ml.	$5{\cdot}8 \times 10^3$	$1{\cdot}5 \times 10^5$	$6{\cdot}9 \times 10^5$

The figures show that the percentage of surviving bacteria increases with the increase of inoculum size. About 100 single colonies were picked and transferred to plain agar, and the resulting cultures transferred to agars containing 1·0, 10·0 and 100·0 μg. streptomycin/ml. The results obtained with the surviving bacteria from the three inocula were very similar:

Inoculum	1	2	3
Total colonies tested	31	33	32
Growing on 1·0 μg./ml.	30	30	28
Growing on 10·0 and 100·0 μg./ml.	1	1	1

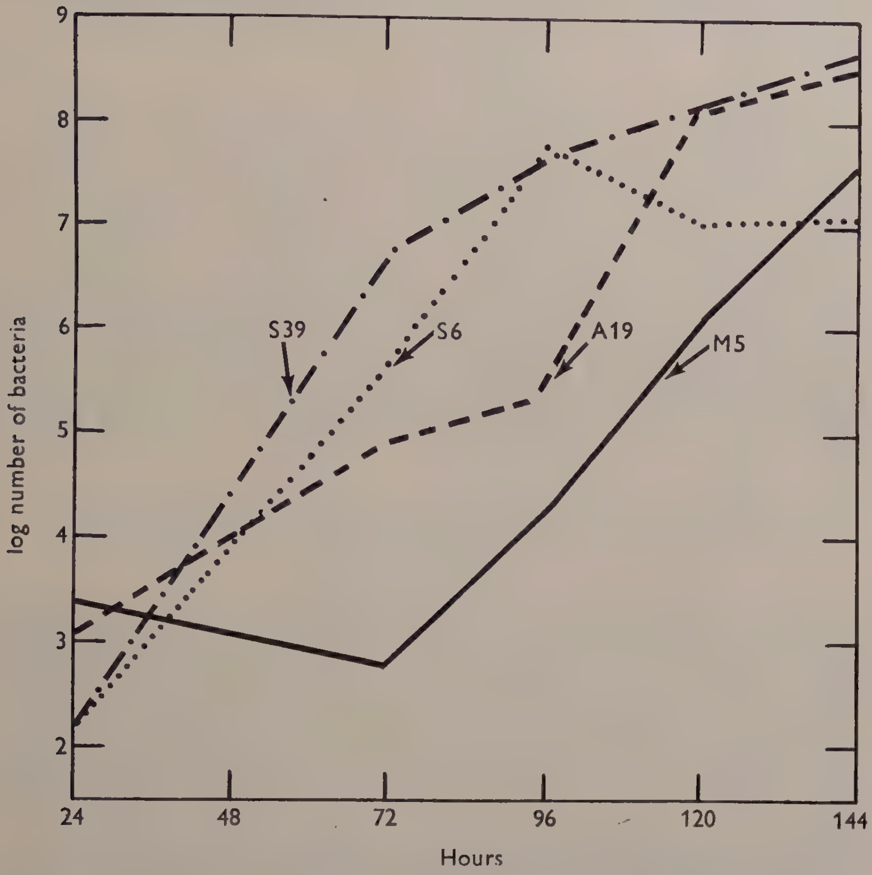

Fig. 3. The changes in bacterial count 24–144 hr. in broth containing 1·0 μg. streptomycin/ml. Development of resistant micro-organisms.

This experiment showed that after 24 hr. the surviving bacteria were nearly all resistant to 1·0 μg. streptomycin/ml., but only about 3 % were resistant to higher concentrations. All bacteria resistant to 10 μg./ml. were resistant to 100·0 μg./ml. Similar experiments were carried out with other strains exposed to different streptomycin concentrations. Most of the strains isolated at 1·0 μg./ml. were resistant only against this concentration. Of 284 strains isolated at 1·0 μg./ml. 17 were resistant against 10 or more μg./ml. When a strain tolerated the tenfold concentration then it also tolerated all higher concentrations up to 1000 μg./ml. On the other hand, all the strains isolated from broth containing 10 and 100 μg. streptomycin/ml. tolerated all the higher concentrations up to 1000 μg./ml.

In these brucella cultures there appear to be present mainly two streptomycin-resistant variants: (1) variants partially resistant to 1 μg./ml. but not resistant to higher concentrations; (2) completely resistant variants resistant to 10 and 100 μg./ml. and higher concentrations. The following experiments were, therefore, carried out at 1·0, 10·0 and 100·0 μg./ml. to determine the MPN of the partially and completely resistant cells present in different cultures.

Determination of the MPN of organisms resistant to 1·0 μg. streptomycin/ml.

To determine the MPN, 24 hr. broth cultures were diluted in tenfold steps and from suitable dilutions 1 ml. volumes were added to tubes containing 9 ml. broth, and streptomycin added in the required concentration. After preliminary experiments to determine after a 5-day incubation period the range of inocula just sufficient to give resistant organisms, three inocula around this critical limit were selected and five broth tubes seeded with each inoculum. The inocula sizes differed by a factor of 10. The MPN was determined after incubation for 5 days for an inoculum ten times greater than the largest inoculum used in the experiment, by means of the tables of Hoskins (1934).

Table 1. *Determination of the most probable number (MPN) of brucella cells resistant to 1·0 μg. streptomycin/ml. in 24 hr. broth cultures*

Strain	Size of inocula			Positive results per 5 portions of each inoculum			MPN	No. of organisms per resistant cell
	1	2	3	1	2	3		
M5	$1{\cdot}8\times10^7$	$1{\cdot}8\times10^6$	$1{\cdot}8\times10^5$	5	0	0	$23/1{\cdot}8\times10^8$	$7{\cdot}8\times10^6$
M7	$3{\cdot}5\times10^7$	$3{\cdot}5\times10^6$	$3{\cdot}5\times10^5$	1	0	0	$2/3{\cdot}5\times10^8$	$1{\cdot}8\times10^8$
S39	$1{\cdot}2\times10^7$	$1{\cdot}2\times10^6$	$1{\cdot}2\times10^5$	4	0	0	$13/1{\cdot}2\times10^8$	$9{\cdot}2\times10^6$
S6	$2{\cdot}6\times10^7$	$2{\cdot}6\times10^6$	$2{\cdot}6\times10^5$	5	0	0	$23/2{\cdot}6\times10^8$	$1{\cdot}1\times10^7$
A19	$1{\cdot}3\times10^7$	$1{\cdot}3\times10^6$	$1{\cdot}3\times10^5$	5	0	0	$23/1{\cdot}3\times10^8$	$5{\cdot}7\times10^6$
A2308	$3{\cdot}2\times10^7$	$3{\cdot}2\times10^6$	$3{\cdot}2\times10^5$	1	0	0	$2/3{\cdot}2\times10^8$	$1{\cdot}6\times10^8$

Table 1 shows the results of a typical experiment. There exist marked differences between the other strains and strains M7 and A2308 which, in the experiment shown in Fig. 3, did not produce resistant organisms since the inoculum was too small. The results in Table 1 explain this previous result. The strains M7 and A2308 had only one resistant cell among at least 10^8; all the other strains had resistant cells in much smaller bacterial populations. The experiment shows that it is possible to determine the MPN of organisms resistant to a certain concentration of streptomycin when the following conditions are fulfilled: (1) the largest inoculum must be great enough to enable the appearance of resistant variants in at least one of five tubes; (2) the smallest inoculum must be small enough to enable resistant variants to appear in less than five tubes. When resistant organisms appear in all five tubes inoculated with the smallest inoculum their number cannot of course be calculated.

Table 2. *Variation with time of the number of cells resistant to* 1·0 *μg. streptomycin/ml. in cultures originating from a single colony*

Strain	Age of culture (days)	MPN	No. of organisms per resistant cell
M 5	1	$23/1 \cdot 8 \times 10^8$	$7 \cdot 8 \times 10^6$
	3	$240/2 \cdot 0 \times 10^9$	$8 \cdot 3 \times 10^6$
	17	$79/1 \cdot 4 \times 10^8$	$1 \cdot 8 \times 10^6$
M 7	1	$2/3 \cdot 5 \times 10^8$	$1 \cdot 8 \times 10^8$
	3	$2/1 \cdot 7 \times 10^8$	$8 \cdot 5 \times 10^7$
	17	$27/4 \cdot 0 \times 10^8$	$1 \cdot 5 \times 10^7$
	25	$22/2 \cdot 0 \times 10^8$	$9 \cdot 1 \times 10^6$
	30	$13/4 \cdot 0 \times 10^8$	$3 \cdot 1 \times 10^7$
S 39	1	$13/1 \cdot 2 \times 10^8$	$9 \cdot 2 \times 10^6$
	3	$2/2 \cdot 1 \times 10^7$	$1 \cdot 1 \times 10^7$
	17	$240/8 \cdot 0 \times 10^6$	$3 \cdot 3 \times 10^4$
S 6	1	$23/2 \cdot 6 \times 10^8$	$1 \cdot 1 \times 10^7$
	3	$9/2 \cdot 2 \times 10^7$	$2 \cdot 4 \times 10^6$
	25	$13/2 \cdot 0 \times 10^7$	$1 \cdot 6 \times 10^6$
	30	$13/8 \cdot 2 \times 10^7$	$6 \cdot 6 \times 10^6$
A 19	1	$23/1 \cdot 3 \times 10^8$	$5 \cdot 7 \times 10^6$
	3	$2/3 \cdot 0 \times 10^6$	$1 \cdot 5 \times 10^6$
	17	$1600/6 \cdot 0 \times 10^6$	$3 \cdot 8 \times 10^3$
A 2308	1	$2/3 \cdot 2 \times 10^8$	$1 \cdot 6 \times 10^8$
	3	$2/1 \cdot 6 \times 10^8$	$8 \cdot 0 \times 10^7$
	25	$180/1 \cdot 6 \times 10^8$	$9 \cdot 0 \times 10^5$

Table 2 shows that the MPN varies with age of culture. The general trend seems to be that in older more dissociated cultures the MPN of resistant variants increases. This increase of resistant cells was very marked in the cultures of the strains S 39, A 19 and A 2308.

Determination of the MPN of resistant cells at 10 *and* 100 *μg. streptomycin/ml.*

Using different inoculum sizes at a constant concentration of streptomycin it was not possible to determine the minimal inoculum necessary to give resistant strains in streptomycin concentrations of 10 and 100 μg./ml. since resistant organisms appeared in many instances in smaller inocula, while relatively large inocula did not give resistant strains. A typical experiment is recorded in Table 3 which shows that, using only one tube for each inoculum, it is impossible to find a clear limit between inocula large enough and those too small to give rise to resistant variants. There exists, therefore, only the possibility of determining the MPN of resistant organisms in the total bacterial population. Since all the strains isolated at 10 μg./ml. were also resistant to all the higher concentrations all the following experiments were carried out at 100 μg./ml. To determine this number the method for the calculation of small numbers of coliform bacteria was used; 10·0, 1·0 and 0·1 ml. of 24 hr. broth cultures were inoculated into broth tubes containing

Table 3. *The appearance of resistant forms in cultures exposed to* 10 *and* 100 μ*g. streptomycin/ml. for* 10 *days at* 37°

Streptomycin (μg./ml.)	Strain	No. of organisms inoculated/10 ml. ($\times 10^6$) 64	160	320	640	1600	3200	8000
10·0	M5	0	0	0	0	R	.	.
	M7	0	0	0	0	R	.	.
	S39	0	0	0	R	0	.	.
	S6	0	0	R	0	0	.	.
	A19	0	0	0	R	0	.	.
	A2308	0	0	0	0	0	.	.
100·0	M5	.	.	0	0	R	0	0
	M7	.	.	0	0	0	0	R
	S39	.	.	R	0	0	0	0
	S6	.	.	0	0	R	0	0
	A19	.	.	R	0	R	0	0
	A2308	.	.	R	0	R	0	0

0 = no appearance of resistant organisms.
R = resistant organisms appeared.

100 μg. streptomycin/ml. For the inoculation of 10·0 ml. of culture, tubes containing 10·0 ml. broth + 200 μg. streptomycin/ml. were used in order to bring the final concentration to 100 μg./ml. Five tubes were used for each size of inoculum. The results are summarized in Table 4 which shows that not all strains were equally able to produce completely resistant variants. Considerable quantitative differences existed and under the conditions of the experiment strain M5 was only once able to produce a resistant variant with an inoculum of 10·0 ml. Strain A19 also showed irregular behaviour; it produced resistant variants with an inoculum of 1·0 ml. in two tubes, but did not produce resistant variants in any of the 5 vol. of 10·0 ml. The ability of the strains M5 and A19 to produce resistant variants was therefore re-examined using larger volumes of broth cultures. Five volumes of 100 ml., 5 of 10 ml. and 5 of 1 ml. were used; concentration streptomycin 100 μg./ml. To the 100 and

Table 4. *The MPN of brucella cells resistant to* 100 μ*g. streptomycin/ml. determined for* 100 *ml. of* 24 *hr. broth cultures*

Five replicate tubes were inoculated with varying amounts, to give three tenfold dilutions.

Strain examined	Volume (ml.) 10·0	1·0	0·1	MPN (resistant cells/100 ml.)	Total no. of organisms/100 ml.	No. of organisms/resistant cell
	No. of positives in 5 portions					
M5	1	0	0	2·0	$3{\cdot}5 \times 10^{10}$	$1{\cdot}8 \times 10^{10}$
M7	1	1	0	4·0	$4{\cdot}5 \times 10^{10}$	$1{\cdot}1 \times 10^{10}$
S39	4	1	1	21·0	$4{\cdot}3 \times 10^{10}$	$2{\cdot}0 \times 10^{9}$
S6	3	1	0	11·0	$4{\cdot}5 \times 10^{10}$	$4{\cdot}1 \times 10^{9}$
A19	0	2	0	3·7	$2{\cdot}6 \times 10^{10}$	$7{\cdot}0 \times 10^{9}$
A2308	4	3	0	27·0	$3{\cdot}1 \times 10^{10}$	$1{\cdot}1 \times 10^{9}$

10 ml. volumes equal volumes of broth + 200 μg. streptomycin/ml. were added to bring the final concentration to 100 μg./ml. This experiment is summarized in Table 5. It is interesting to note that the figure obtained in this experiment for the strain M5 was the same as obtained previously (cf. Table 4). The figure for A19 was about twice that obtained in the previous experiment. The MPN of cells resistant to 100 μg./ml. remained fairly constant at the different periods of incubation, although in this case also the general trend seemed to show an increase of resistant cells with the age of culture (Table 6).

Table 5. *The most probable numbers of brucella cells resistant to* 100 μg. *streptomycin/ml. determined for* 1000 *ml. of broth cultures of strains M* 5 *and A* 19

Five portions of each volume, 100, 10 and 1 ml., were seeded.

Strain	Volume (ml.) 100·0	10·0	1·0	MPN resistant organisms/ 1000 ml.	Total no. of organisms/ 1000 ml.	No. of organisms/ resistant cell
	No. of positives in 5 portions					
M5	4	1	1	21·0	3·8 × 10¹¹	1·8 × 10¹⁰
A19	3	1	0	11·0	2·6 × 10¹¹	2·4 × 10¹⁰

Table 6. *Variation in the number of cells resistant to* 100 μg. *streptomycin/ml. in cultures originating from a single colony*

Strain	Age of culture (days)	MPN	No. of organisms/ resistant cell
M7	3	$4{\cdot}0/6 \times 10^{10}$	$1{\cdot}5 \times 10^{10}$
	30	$10{\cdot}0/4 \times 10^{8}$	$4{\cdot}0 \times 10^{7}$
A19	3	$4{\cdot}0/3 \times 10^{10}$	$7{\cdot}5 \times 10^{9}$
	17	$23{\cdot}0/12 \times 10^{9}$	$5{\cdot}2 \times 10^{8}$
A2308	3	$1{\cdot}8/25 \times 10^{9}$	$1{\cdot}4 \times 10^{10}$
	17	$27{\cdot}0/31 \times 10^{8}$	$1{\cdot}1 \times 10^{8}$
	30	$4{\cdot}5/8 \times 10^{7}$	$1{\cdot}8 \times 10^{7}$

Agreement between the determination of the MPN with other methods

The figures obtained by the MPN method were compared with the results of direct observation of resistant cells when large quantities of culture were plated out in the presence of 100 μg. streptomycin/ml. The method was compared with the centrifugation method in which the whole bacterial growth from 100 ml. of culture was collected on a refrigerated centrifuge and poured in plates containing 100 μg. streptomycin/ml., and with a simple plating method in which only 2·0 ml. of culture were removed and plated with the same concentration of streptomycin. A comparison was made between the growth of a strain known to be resistant to 100 μg. streptomycin/ml., starting from an inoculum of about 5 cells/100 ml., and the growth of the resistant cells surviving from a 48 hr. non-resistant culture to which 50 % (v/v) of fresh broth and 100 μg. streptomycin/ml. were added.

Table 7 shows that the results of the MPN method agree with those obtained by the centrifugation method until the upper limit of 1600 cells/100 ml. of broth culture is reached. The direct plating method does not give reliable results so long as the number of resistant cells is lower than 100/100 ml. With an increase of number of resistant organisms above 1000 the direct plating methods without or with suitable dilutions is the method of choice. The experiment presented in Table 7 also shows that in the non-resistant S39 strain, resistant bacteria were present in the culture at the time when streptomycin was added and that these bacteria multiplied slowly after a prolonged lag phase which lasted about 14 hr.

Table 7. *Streptomycin resistant cells appearing in a culture of a non-resistant strain exposed to* 100 *µg. streptomycin/ml., and from the culture of a resistant strain exposed to the same concentration of streptomycin*

Strain	Time (hr.)	No. of resistant bacteria determined by pouring plates with 100 µg. streptomycin/ml. and		Determination of MPN/100 ml. broth culture			
		2 ml. culture	the sediment of 100 ml. culture	10·0	1·0	0·1	MPN
S38 non-resistant	0	0	16	4	2	0	22
	10	0	23	5	1	0	33
	14	0	26	5	2	0	49
	18	2	59	5	1	0	33
	22	2	78	5	4	0	130
	34	22	340	5	5	3	920
	58	890	12,000	. .	.	.	.
S39 resistant	0	0	5	3	0	0	7·8
	10	4	124	5	4	0	180
	14	14	278	5	5	3	920
	18	80	1,550	5	5	4	1,600
	22	265	5,560	5	5	5	.
	34	46,000*	960,000*	5	5	5	.

* By the aid of dilutions.

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Variation in Penicillin Resistance in Single-cell Cultures of *Staphylococcus aureus*

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SUMMARY: Using staphylococci grown from single-cell isolations made at various stages of selection on penicillin media, it was observed that the 18 hr. progeny show variations in penicillin sensitivity, as tested by increase in resistance. The range of variation is about the same at any stage in selection; variation appears to be continuous in any one population of the microbes, since the number showing increased resistance depends upon the closeness of the steps in penicillin concentration used in testing the resistance.

There does not yet appear to be sufficient evidence on the frequency of variation in pure strains of organisms; the following investigations were undertaken to discover whether penicillin resistant variants appear in strains grown from single cells and in which therefore no possibility of crossing between genetically different individuals, such as was described by Tatum & Lederberg (1947), exists.

EXPERIMENTAL

A strain of coagulase-positive *Staphylococcus aureus* was obtained from a previously unopened abscess in a patient who had never been treated with penicillin. A single colony from the primary growth was subcultured and a second well-isolated colony selected as the parent strain of the series. From a young culture of this strain P two single cocci were isolated by the micromanipulator to form the first generation; these are I and II respectively (Fig. 1). The three strains P, I and II were identical by phage-typing (Williams, 1950).

Method 1

The three strains were examined for penicillin sensitivity by seeding on to a series of agar media containing concentrations of penicillin increasing by $\frac{1}{400}$ unit/ml. The daughter strains I and II differed in penicillin sensitivity by this amount, I being as resistant as P and II more resistant than either. The difference was shown not only by the end-point of the titration, but also by colony size with partially inhibitory penicillin concentrations; the colonies of II were larger than those of I.

With implants of about 100 organisms the titration gave a sharp end-point; there was no evidence of scatter of sensitivity that could not be accounted for by the experimental error of the method. When however the concentrations of penicillin differed by only $\frac{1}{1200}$ unit/ml. from one plate to the next, small but real differences in sensitivity were observed in colonies from one inoculum.

When the size of implant was increased considerably some cells survived and grew at higher concentrations of penicillin than was the case with the small inoculum. This phenomenon has long been known (see Florey, 1949), but in the present experiments a regular relationship between the size of implant and the number of colonies developing was also noted, namely, that for every tenfold increase in implant there was a 40 % increase of resistance as judged by the final concentration on which any colonies appeared. Quantitative tests by a tube method were made to confirm this finding.

Method 2

The tube method was based on that used for presumptive coliform count (Ministry of Health, 1939) and at first the McCready tables, suitably modified, were used. As, however, more data were available than is usual in coliform tests the number of survivors was calculated from table VIII, 2, of Fisher & Yates (1948). The test consisted of quintuplicate titrations, using eight serial tenfold dilutions of an 18 hr. broth culture, made from a stock kept at 4° in Robertson's meat medium following its isolation and growth from the single cell. To avoid clumps the suspension was shaken mechanically and then allowed to stand, the upper layers being taken for use. To 0·05 ml. lots of the eight dilutions ($10^0 - 10^{-7}$) 2·5 ml. of the penicillin solution in broth was added and each of the mixtures was then distributed among the five tubes forming a quintuplicate set with 0·01 ml. of the suspension in 0·5 ml. penicillin solution in each. The cultures were tested with eleven penicillin concentrations and one control series in broth alone. The number of organisms in the inoculum was estimated by plate counts. It would be possible to dispense with this if the culture were serially diluted so as to be sterile at the end of the series, since the calculation is most accurate when there are groups of tubes without growth.

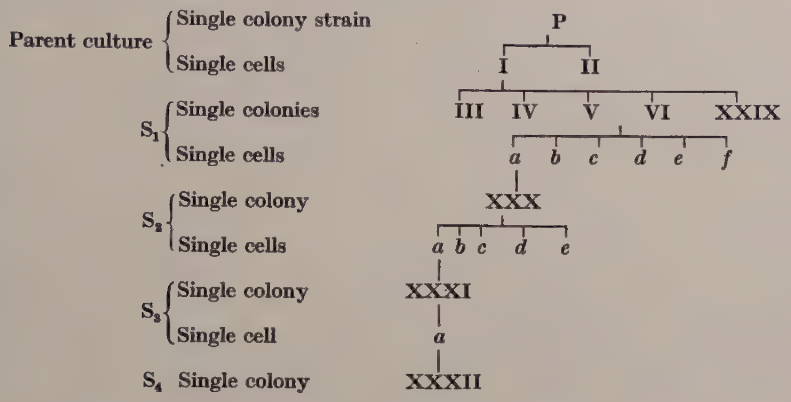
Table 1 shows one complete titration and column 10 the frequency of

Table 1. *Specimen tube titration showing variations in sensitivity of single-cell strain XXXa of* Staph. aureus

Penicillin concentration (u./ml.)	Dilutions of culture								Estimated no. of survivors/ 10^6 implanted
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
	No. of tubes positive/group of five								
0·000	.	.	.	.	5	5	5	5	.
0·125	.	.	.	.	5	5	5	5	.
0·15	.	.	.	.	5	5	5	5	.
0·2	.	.	.	.	5	5	5	5	.
0·25	.	.	.	.	5	5	5	5	.
0·375	.	.	.	.	5	5	5	5	.
0·5	.	.	.	.	5	5	5	5	.
0·625	.	.	.	5	5	5	5	3	290,000
0·75	.	.	5	5	5	3	1	0	44,000
0·875	.	5	5	5	3	0	0	0	285
1·0	5	5	5	5	0	0	0	0	66
1·25	5	5	4	2	1	0	0	0	77
Inoculum estimated	26×10^6	26×10^5	260,000	26,000	2,600	260	26	4·25	

survivors/million implant. It is to be noted that here, as in Demerec's work (1948), the penicillin is used to detect variation in resistance and not to induce it.

Further subcultures of the survivors were made, and grown without penicillin; from these cultures single cells were picked and the distribution of the sensitivities determined in the populations derived from them. The derivation of the strains was as follows:



The results with four single-cell cultures, one from each subculture, are shown in Fig. 2 the log number of survivors being plotted against the log dose of penicillin. The curves are approximately linear and parallel. Usually several separate cells were taken in each of the generations and all gave the same type of curve; but the position of this curve varied considerably with each single coccus even in the same subculture.

The findings recorded in Fig. 2 suggest that there is a definite ratio by which the penicillin resistance increases in each subculture. But the truth of this deduction is restricted to the conditions of the experiment, since it is by the selection of the most resistant members of the population that this result is achieved. There have been in each subculture cells which were less resistant than those whose resistance is recorded in the graph.

The finding that a bacterial cell has survived exposure to a certain concentration of penicillin is no guarantee that single cells picked from its progeny will give populations of the same or greater resistance; as soon as growth is allowed to take place on normal media the variation of sensitivity will be resumed in both directions and thereafter a cell that is less resistant than the parent may be picked instead of a more resistant one. Variants of diminished resistance are quite common; e.g. of 15 single-cell strains derived directly or indirectly from strain I three more sensitive than the cell giving rise to the colony from which they were picked. Two of these were more sensitive than any in the previous generation; they were in fact sensitive to 0·075 unit/ml. as compared with the colony from which they came (2·0 units/ml.) and with the previous subculture (0·125 unit/ml.). The strains were tested for extracellular penicillinase production but this had not developed in any of them.

DISCUSSION

From the evidence it is clear that single cells give rise, on overnight culture, to populations containing individuals differing to a considerable degree from the parent. For one characteristic, penicillin sensitivity, variation is of the same sort whether the strain has been exposed to the drug or not; the population derived from strain I, as characterized by the survivor dose curve, is similar to that from strain XXXI which had been selected three times for increased resistance.

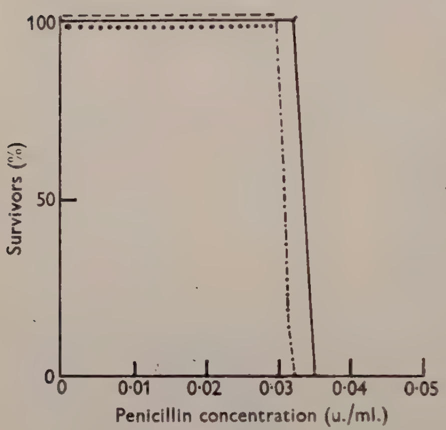


Fig. 1

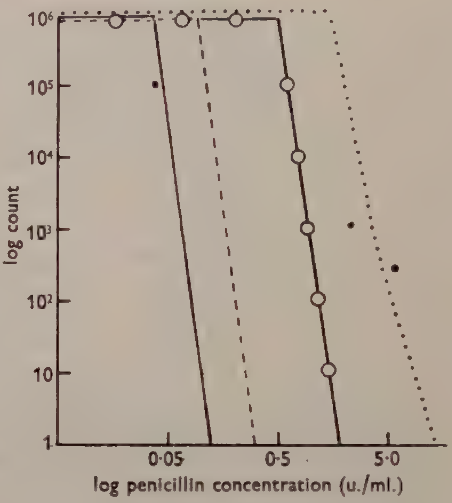


Fig. 2

Fig. 1. Titration of sensitivity of single-cell strains of staphylococci using small implants. Key to curves: – – –, parent strain; · · · · ·, single-cell strain I; · – · – ·, single-cell strain II. Parent strain and strain I give a value of 0·0325 u./ml. for 0 % survivors. Strain II gives a value of 0·035 u./ml. under identical conditions.

Fig. 2. Results of tube titration of sensitivity of single-cell strains of staphylococci. Key to curves: ———, strain I; – – –, strain VI*a*; —○—, strain XXX*a*; · · · · ·, strain XXXI*a*. The four subcultures show a similar distribution of sensitivity, although the average sensitivity of each is at a different level. Each curve on this graph is built up from 11 separate points, the only 3 points not falling on their line are marked ●.

The term used to describe this type of variation needs careful consideration. They do not form a genetically uniform clone. The term mutation is also applied to the kind of variation we are considering. Demerec (1948) cautiously suggested that the rate of mutation to resistance might be of the order of one in 10^8 cells.

Such a resistant variant can be selected, but if it should be styled a mutant then so should strain II which differs from the P and I strains by only $\frac{1}{400}$ of a unit; so too should each of the five cells forming the third series (XXX, *a*–*e*), all of which differed from the parent of the colony from which they were isolated and only two of which had the same resistance. In the plate method of titration, differences of up to 10 % in the count may be due to sampling error and an error of this size would easily obscure fine differences. It can be inferred from the populations raised from the single cells that about this proportion of cells are detectably more resistant than the average.

The method of recording results on a log scale, necessitated by the size of the populations, obscures any change at the 'more sensitive' part of the scale. It cannot, therefore, be proved whether there are as many individual cells more sensitive than the average as there are more resistant ones. Yet many such individuals are present, for they may be picked accidentally from a population, the mean resistance of which is high. Also where the parent cell has been isolated in the presence of particular concentrations of penicillin and not from a culture in normal media, the sensitivity of that single cell is likely to be resistant to the 'isolating' concentration, yet the population derived from it contains cells sensitive to less than this concentration. No method exists, however, for detecting very rare variants with greatly increased sensitivity.

Similar high rates have been reported for the mutation of H antigens in *Salmonella typhimurium* (Stocker, 1949). Deskowitz & Shapiro (1935) found an R variant giving 17 % S colonies on solid medium.

Segregation on strict mendelian lines as an explanation of this variation also presents difficulties; if we start with a single cell and it is assumed that no new genetic material can be introduced, any redistribution of nuclear material must be by autogamy. Even if the cell were heterozygous the repeated reselection employed would give a uniform population by the fourth subculture.

If we are to be driven to use the term mutation then we are using it in a sense not usually employed by workers in other fields and have to accept a rate of mutation of as high as one in ten. Whatever we may call the variation it is continuous and not discontinuous for the particular characteristic studied, and by the end of the thirty generations, represented by the 18 hr. cultures sampled in these experiments, a regular distribution has been reached. It may well be that the exact interpretation will depend on the correlation of these and similar results with the cytological studies of microbial division and on breeding experiments like those of Tatum & Lederberg (1947) and Zelle & Lederberg (1951).

One other conception must be examined in the light of these results. Superficially the upper parts of the curves in Fig. 2 and the whole of Fig. 1 closely resembles Demerec's figures (1948), though he did not start with single cells but used a stock strain; nevertheless, these two figures are to some extent artefacts and so by implication is the idea of stepwise variation. The size of the step is limited only by the practicable implant and not by any inherent characteristics in the cells. The number of steps that could be interposed seems almost limitless, since, once the critical level has been reached where any members of a population are inhibited, a small increase in penicillin concentration will decrease the numbers of survivors, and from these, populations can be raised which differ from the original parent strain either by little or much, depending on the exact point at which selection was made. It seems clear that within the individual staphylococcus is a capacity for variation much greater than has been supposed. In its flexibility it behaves in a way not anticipated either from simple fission into two identical units or from the laws of inheritance postulated for plants and animals.

My thanks are due to Dr A. Voureka for providing single-cell cultures for two of the generations of staphylococci and to Dr R. E. O. Williams for the phage typing.

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The Effect of Thiosemicarbazide and other Urea Analogues on the Growth of *Tetrahymena geleii*

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SUMMARY: A number of urea analogues have been tested for inhibitory effects on the growth of *Tetrahymena geleii.* Semicarbazide and thiosemicarbazide appear to interfere with the formation of compounds containing carbonyl-groups. The inhibition can be partially overcome by increasing the concentration of pyridoxamine in the medium or completely overcome by adding keto-acids.

The thiosemicarbazones of β- and γ-pyridine carboxaldehyde and of *p*-acetaminobenzal are inhibitory but reversal of this inhibition was not possible with the compounds used.

Interest in thiosemicarbazide and the thiosemicarbazones for the treatment of tuberculosis was aroused by the report of Domagk, Behnisch, Mietzsch & Schmidt (1946). It was thought possible that the ciliate *Tetrahymena geleii* might serve as a test organism for further study of these compounds. This ciliate has the advantage of resembling the mammal in its metabolic requirements and therefore might be useful for the toxicity studies. On the other hand, its faster growth rate would be desirable as compared to the mycobacteria.

It was proposed therefore to determine the toxicity to *T. geleii* of thiosemicarbazide and a number of related substituted ureas, and if possible to obtain reversal of the toxic effects, in order to gain some insight into the metabolic system or systems involved.

MATERIAL AND METHODS

The organism used was *T. geleii* W. It was grown in medium A (Dewey, Parks & Kidder, 1950) or medium A in which Tween 80 (10 mg./ml.) was substituted for Tween 85. Appropriate omissions were made from these media. The experiments were carried out in such a way that the urea analogues were autoclaved separately from the remainder of the medium. The concentrations of these compounds were varied over a range so that half maximal inhibition of growth occurred at an approximately median concentration of the inhibitor (Kidder, Dewey & Parks, 1951). The growth responses were determined turbidimetrically, after 96 hr. incubation at 25° in tubes held in a slanted position, by use of a Lumetron photoelectric colorimeter with filter no. 650.

The compounds used, with the exception of hydroxylamine, are all related to urea (Fig. 1): ethyl carbamate, urea, thiourea, nitrourea, acetylurea, acetylmethylurea, 3-thiosemicarbazide, semicarbazide, glucose thiosemicarbazone, pyruvic acid thiosemicarbazone, *p*-acetaminobenzal thiosemicarbazone, the β- and γ-pyridine carboxaldehyde thiosemicarbazones and pyridoxal thiosemicarbazone. The following compounds (Fig. 2), either because of their structural similarity or because of their known or possible metabolic functions, were tested for their ability to overcome inhibition: creatine, guanidoacetic acid, guanidine, aminoguanidine, pyruvic acid, α-ketoglutaric acid, γ-aminobutyric acid, pyridoxal, pyridoxal phosphate, pyridoxamine and nicotinamide.

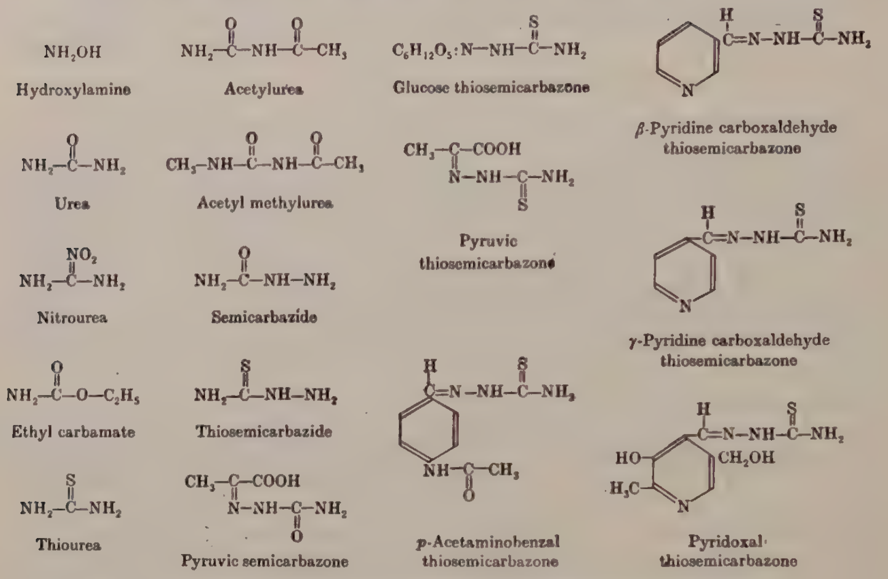

Hydroxylamine
Acetylurea
Glucose thiosemicarbazone
β-Pyridine carboxaldehyde thiosemicarbazone
Urea
Acetyl methylurea
Pyruvic thiosemicarbazone
Nitrourea
Semicarbazide
γ-Pyridine carboxaldehyde thiosemicarbazone
Ethyl carbamate
Thiosemicarbazide
Thiourea
Pyruvic semicarbazone
p-Acetaminobenzal thiosemicarbazone
Pyridoxal thiosemicarbazone

Fig. 1. Structures of thiosemicarbazide and related compounds tested for toxicity to *T. gelii*.

RESULTS

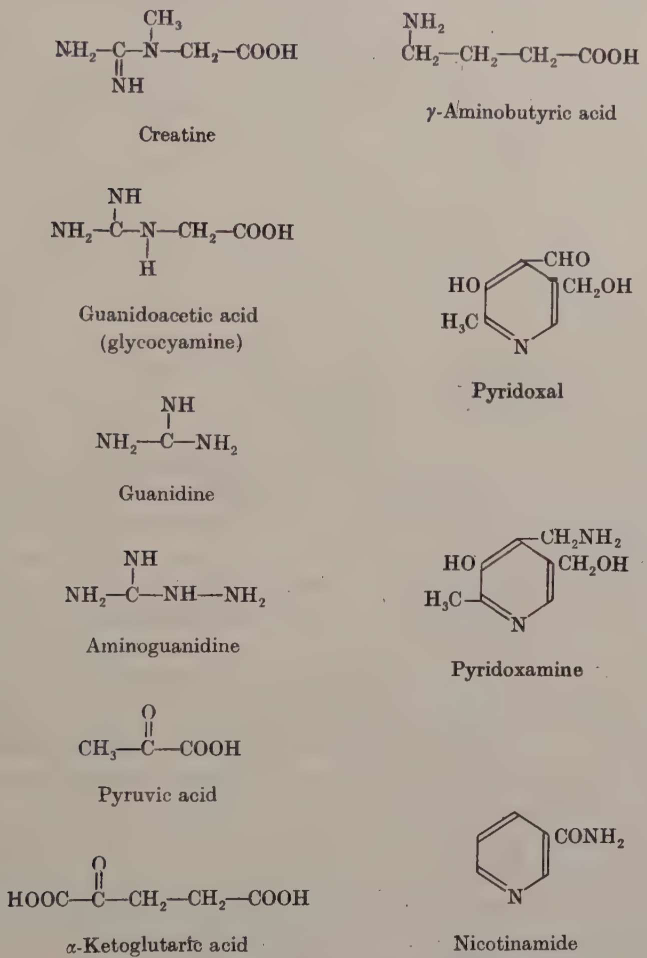
Table 1 lists the compounds tested and gives the amount required for half-maximal inhibition in complete basal medium (which contains 0·1 μg. of pyridoxal HCl and of pyridoxamine HCl/ml.). It may be seen that the compounds most effective in the treatment of tuberculosis (Levaditi, Gerald,

Table 1. *Inhibition of* T. geleii *by urea and analogues*

Compound	Amount required for ½ maximum inhibition (μg./ml.)	Compound	Amount required for ½ maximum inhibition (μg./ml.)
Urea	412	Pyruvic semicarbazone	2000
Nitrourea	123	Pyruvic thiosemicarbazone	600
Ethyl carbamate	5250	Glucose thiosemicarbazone	1650
Thiourea	1120	β-Pyridine carboxaldehyde thiosemicarbazone	12
Acetylurea	3100	γ-Pyridine carboxaldehyde thiosemicarbazone	21
Acetylmethylurea	2840	p-Acetaminobenzal thiosemicarbazone	100
Hydroxylamine	12	Pyridoxal thiosemicarbazone*	No inhibition
Semicarbazide	126		
Thiosemicarbazide	123		

* Replaced vitamin B_6 with 10–15 % activity.

Vaisman & Ray, 1950) were those which were the most inhibitory to *T. geleii*. No inhibition index can be given, of course, since the system in which these compounds are acting is unknown. The various members of the vitamin B_6 group (Table 2) were effective in reversing the inhibition caused by semicarbazide and thiosemicarbazide but not that of the other compounds tested. It should also be noted that thiosemicarbazide reversal was only partial. For example, after a certain concentration of pyridoxamine is reached, further increases in concentration give no further decrease of inhibition. Of the remainder of the compounds listed above as having been tested for their effect in reversing toxicity, only the keto-acids were active.



Creatine

γ-Aminobutyric acid

Guanidoacetic acid (glycocyamine)

Pyridoxal

Guanidine

Aminoguanidine

Pyridoxamine

Pyruvic acid

α-Ketoglutaric acid

Nicotinamide

Fig. 2. Structures of various compounds tested for ability to overcome toxicity of thiosemicarbazide and related compounds for *T. gelii*.

Hydroxylamine differed from the semicarbazides in that inhibition by it was overcome by the keto-acids but not by members of the vitamin B_6 group.

Table 2. *Effects of* B_6 *vitamins on inhibition by various urea analogues*

B_6 vitamin additions (μg./ml.)	Urea analogue inhibitors: Urea	Nitro-urea	Ethyl carbamate	Semi-carbazide	Thiosemi-carbazide	Hydroxyl-amine	β*	γ†
	Amounts required for ½ maximum inhibition (μg./ml.)							
				Pyridoxamine				
0·005	410	126	5500	46	22	9·5	9·7	15·4
0·05	455	141	5500	166	145	12·2	11·7	24·5
0·5	.	.	.	.	159	9·3	11·4	27·9
2·0	.	.	.	.	159	.	.	.
				Pyridoxal				
0·005	.	.	.	.	35	.	8·8	15·0
0·05	.	.	.	.	153	.	7·8	17·2
				Pyridoxal phosphate				
0·02	.	.	.	.	13	5·0	.	.
0·2	.	.	.	.	54	9·2	.	.
2·0	.	.	.	.	147	9·2	.	.

* = β-Pyridine carboxaldehyde thiosemicarbazone.
† = γ-Pyridine carboxaldehyde thiosemicarbazone.

It is also of interest that aminoguanidine, which has been suggested as being active in the metabolism of formaldehyde (Bernheim, 1950), was itself somewhat inhibitory and enhanced the inhibition produced by the semicarbazides.

The inhibition due to the semicarbazones was not affected by increasing the concentration of B_6 compounds in the medium beyond the slight effect obtained by the increased growth in the presence of the larger amounts of the vitamin. The keto-acids were also ineffective in overcoming the inhibition caused by these compounds, as was nicotinamide, which may be considered to be a structural analogue of the β-pyridine carboxaldehyde thiosemicarbazone.

In the belief that the pyridine carboxaldehyde thiosemicarbazones might be acting as structural analogues of pyridoxal, the thiosemicarbazone of pyridoxal was prepared. This compound proved to be completely uninhibitory. When tested in the absence of vitamin B_6 it promoted growth. This was taken as evidence that the organism is capable of hydrolysing the thiosemicarbazone with consequent release of pyridoxal. The thiosemicarbazone of pyridoxal had 10–15 % activity on a molar basis as compared to pyridoxal. The activity tended to decrease as the amount of thiosemicarbazone was increased. The thiosemicarbazone of *p*-acetaminobenzaldehyde was also tested for growth-inhibiting capacity, but because of its insolubility no precise figure was obtained.

DISCUSSION

Hydroxylamine was included in this series of compounds because, like the semicarbazides, it reacts with carbonyl groups. Whether the oximes formed are, like the semicarbazones, also inhibitory to growth was not determined. Hydroxylamine has, in addition, the ability to form hydroxamic acids with esters and anhydrides and, according to Borek, Grossowicz & Waelsch (1950), it may also produce inhibition by blocking transamidation reactions involving ammonia, asparagine and/or glutamine. The inhibition of bacterial growth due to interference with these reactions occurs at lower concentrations of the inhibitor than the interference with the metabolism of the carbonyl compounds. However, in the case of *T. geleii*, neither ammonia nor asparagine had any effect on the inhibition produced by hydroxylamine. Since the ciliate is said (Seaman, 1950) to produce no acetyl phosphate, the only known site of action for hydroxylamine is in carbonyl metabolism. In this regard it is of interest that acetaldehyde can function as the two-carbon fragment in the metabolism of *T. geleii* (Seaman, 1950) and that hydroxylamine inhibition is readily reversed by keto-acids. On the other hand, vitamin B_6 is without effect on the toxicity of hydroxylamine, in contrast to the case of the semicarbazides.

Pyridoxal phosphate is reported to participitate in the decarboxylation of glutamic acid to γ-aminobutyric acid (Roberts & Frankel, 1951). Since the latter compound was ineffective in overcoming inhibition, it is apparent that thiosemicarbazide is not interfering with the activity of pyridoxal phosphate, *per se*, although it has not been demonstrated that γ-aminobutyric acid is a metabolic product of *T. geleii*. It is also true that creatine and its precursors have not yet been shown to be concerned in the metabolism of *T. geleii* and it is therefore unprofitable to speculate on their relationship to the semicarbazides. It is evident, however, that semicarbazide and related compounds are not acting as structural analogues of guanidine, guanido-acetic acid or creatine, since the latter substances were not active in overcoming inhibition.

It is apparent that the inhibitory effects of the semicarbazides in the growth of *T. geleii* are produced by the ability of these compounds to react with carbonyl groups, since pyruvic and α-ketoglutaric acids overcame the inhibition. Substances containing carbonyl groups appear to be produced during growth and to be necessary for growth. The addition of pyridoxamine in excess of the minimum amount required for growth stimulates the production of keto-acids by *T. geleii* (unpublished data). This effect, however, reaches a maximum, and further increases in pyridoxamine concentration do not influence keto-acid production. It may be assumed that the enzymes responsible for the production of such acids (e.g. deaminases) become saturated with the pyridoxamine coenzyme at this level. This would explain the failure of pyridoxamine to overcome completely the toxicity of the semicarbazides, while pyruvic and α-ketoglutaric acids are effective. This must be regarded as a non-specific effect due to the chemical interaction of these acids with the semicarbazides.

While these observations were responsible for the use of pyridoxamine in overcoming the toxicity of thiosemicarbazide in mice (Parks, Kidder & Dewey, 1951), the mechanism of this action in the latter case appears to be somewhat different, since the keto-acids are inactive. In view of this fact and of the fact that the toxicity of the semicarbazones is not overcome either by pyridoxamine or by keto-acids in *T. geleii*, it will be of interest to determine whether or not pyridoxamine will affect the toxicity of the thiosemicarbazones in mice.

We are indebted to Dr John Aeschlimann of the Hoffmann-LaRoche Co. for samples of *p*-acetaminobenzal thiosemicarbazone and of the two pyridine carboxaldehyde thiosemicarbazones. The pyridoxal phosphate was obtained through the courtesy of Dr Richard F. Phillips of Merck and Co.

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Biochemical Methods for Bacteriology

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SUMMARY: A series of biochemical methods using heavy suspensions of organisms and chemically-defined solutions are described; they include fermentations, reduction of nitrate and of methylene blue, production of indole, hydrogen sulphide and acetoin, and hydrolysis of gelatin, starch and urea. The tests use the preformed enzymes of the bacterial cells and the results are not complicated by side effects or the multiple reactions that occur in cultures growing in a nutrient medium containing the test substrate.

Methods developed by biochemists are seldom used in the characterization of bacterial species. Bacteriologists' tests are normally made in undefined media and the end-result often is a balance of opposing reactions; this applies particularly to the fermentation tests in which acid production from the breakdown of 'sugar' must exceed alkali produced from the breakdown of protein constituents. Davis (1939) added washed bacterial suspensions to powdered sugars and showed that these non-multiplying suspensions gave clearer acid production than the same organisms growing in a nutrient medium containing the sugar. We applied the principle of testing for preformed enzymes in washed suspensions to other biochemical tests and, to avoid the need for massive growths, developed methods using small volumes of reagents.

Micromethods have recently gained favour with bacteriologists (Arnold & Weaver, 1948; Cook, 1948; Elek, 1948; Hannan & Weaver, 1948; Brough, 1950; Galton, Hardy & Mitchell, 1950; Morse & Weaver, 1950; Bachmann & Weaver, 1951; Fabrizio & Weaver, 1951; Hargrove & Weaver, 1951), but nearly all depend on a heavy inoculum and the growth of the organisms in a small volume of medium containing the test substrate. In the development of our methods speed has not been the determining factor, but a reasonably quick result has advantages in that a sterile technique is unnecessary. Chemically clean glassware is essential.

METHODS

Suspensions

Bacteria were grown on a favourable solid medium, preferably without blood or fermentable substance. Incubation was at the optimal temperature and for the shortest time (usually 18–24 hr.) to produce a good growth. A suspension was made by washing the growth from the slope or plate in tap water; this was spun to deposit the cells, and the deposit resuspended in a small volume of water. Suspensions made from blood agar cultures were washed once to remove haemoglobin. For a series of 20 'capillary sugars' and the full range of biochemical tests to be described, 1–1·5 ml. of heavy suspension was required. The number of slopes or plates of each culture incubated depended on the amount of harvested cells to be expected and the number of tests to be made. Nutrient agar slopes, 100 mm. long in 150 × 20 mm. tubes, were used for most organisms; 1–2 slopes of vigorously growing bacteria such as coliforms, pseudomonads and staphylococci normally provided ample growth, but 3–10 slopes or 1–4 plates of serum or blood agar were needed for organisms such as *Streptococcus pyogenes*, *Clostridium tetani* and *Corynebacterium hofmannii*.

Effect of suspension concentration and age of cultures

Suspensions were made as dense as the growth would allow, but there was a minimum opacity equivalent to a total count of *c.* 1×10^{11} cells/ml. below which the reactions became slower, weaker or uncertain. Dilution of the suspension and the age of the culture used in its preparation affect the speed and intensity of reactions. This is illustrated by the results obtained with *Pseudomonas hydrophila* (Table 1). Viable counts (Miles & Misra, 1938) on *Ps. hydrophila* suspensions showed that 10^8 organisms/ml. were inactive; from

Table 1. *Effect of (a) age of culture and (b) suspension concentration on glucose fermentation and biochemical tests shown by* Ps. hydrophila (NCTC 7810)

Test	Time of reading (hr.)	Age of culture								
		24 hr.			48 hr. Concentration of suspension			72 hr.		
		10^{12}	10^{11}	10^{10}	10^{12}	10^{11}	10^{10}	10^{12}	10^{11}	10^{10}
		Acid formation								
Glucose fermentation	1	A	A	0	A	0	0	0	0	0
	2	A	A	0	A	A	0	0	0	0
	4	A	A	A	A	A	0	A	0	0
	24	A	A	A	A	A	A	A	A	A
		Score in test*								
Indole formation	½	6	3	1	6	3	0	3	2	0
	1	8	8	3	6	6	1	6	3	0
	2	10	10	3	10	10	3	10	6	1
Nitrate reduction to nitrite	½	10	3	0	8	0	0	2	0	0
	1	10	3	2	8	1	0	3	0	0
Acetoin formation	½	3	0	0	1	0	0	0	0	0
	1	6	0	0	3	0	0	0	0	0
	2	8	2	0	8	0	0	0	0	0
	4	10	6	0	10	1	0	3	0	0
H_2S formation	1	6	0	0	3	0	0	2	0	0
	2	6	0	0	6	0	0	2	0	0
	4	8	0	0	8	0	0	3	0	0
Gelatin hydrolysis	4	8	6	0	8	6	0	6	3	0
	24	10	10	3	10	10	0	10	10	0
Starch hydrolysis	4	0	0	0	0	0	0	0	0	0
	24	8	6	0	6	3	0	6	6	0

A = acid formed.

* Numbers represent relative strength of reaction: 10 = maximum; 3 = definite positive; 2 and 1 = trace reactions. 0 = no reaction.

this it is clear that unsterilized glassware and tap water are not likely to introduce false reactions.

Effect of medium. The medium on which the culture is grown is important only if it encourages the development of an adaptive enzyme or if it inhibits the production of certain enzymes. A strain of *Bacterium dispar* took 8 days to ferment 1 % lactose in peptone water; suspensions made from growth on nutrient agar took 48 hr. to ferment 5 % lactose in our microtest, but suspensions made from lactose agar fermented lactose in 24 hr. The effect of growth on glucose agar will be considered in connexion with the tests for acetoin and indole.

Buffer is desirable to prolong enzyme action but excess will slow the reactions; 0·025 M-phosphate buffer was found to be the optimal concentration for those tests in which this effect was studied.

Volumes were measured with dropping pipettes (Donald, 1913) but are only approximate as allowance was not made for the variation in drop-size of different solutions.

FERMENTATIONS

Three methods were tried and each showed that most bacteria have greater fermentative ability than is shown by the usual cultural tests in nutrient sugar media. Unfortunately, the three ways of testing our suspensions for preformed fermentative enzymes do not give identical results. Each method gives consistent results but only an extended trial and further experimental work will show which gives the best indication of the enzymic capacity of the test organism.

'*Sugar' solutions.* Glucose, xylose, lactose, sucrose, maltose and glycerol were made up as 25 % (w/v) solutions. Sugars with lower solubilities were made up as follows: galactose, 20 %; raffinose, 10 %; starch, dextrin, mannitol, dulcitol and salicin, 5 %.

Buffer-indicator solution (BI). To 20 ml. 0·025 M-phosphate buffer (pH 6·8) was added 1·0 ml. 1 % ethanolic bromcresol purple. Both sugar solutions and buffer-indicator solutions were sterilized by momentary autoclaving (Davis & Rogers, 1939) or filtration.

Sugar-buffer-indicator solutions (SBI). Equal volumes of sugar solution and buffer-indicator solution were mixed in 65 × 10 mm. tubes. Mixtures were made up each week and when not in use were stored at 4°.

Method 1. Suspension (0·2 ml.), sugar solution (0·1 ml.), and BI (0·2 ml.) are mixed in 65 × 10 mm. tubes, and acid production observed after varying times in a 37° water-bath.

Method 2. Suspension and SBI are mixed in approximately equal volumes in a capillary tube 10 cm. long; one end of the capillary is sealed and the other stuck in plasticine. Readings are made at intervals after incubation at 37°.

Method 3. Durham's tubes (35 × 8 mm.) are half-filled with 1 % New Zealand agar + 0·1 % bromcresol purple. These columns are stored at 4° until used. Two drops (0·04 ml.) sugar solution are pipetted on to the surface of each column, and 0·02 ml. suspension added. Columns are incubated at 37° and read up to 24 hr.

CAPILLARY TESTS

The next three tests were made in capillary tubes 10 cm. long. The tests are simple to carry out and to read, and comment seems unnecessary.

Catalase

'Ten vol.' H_2O_2 is run into a capillary tube, followed by suspension. Gas is usually evolved immediately and only tubes not showing gas within 10 sec. are sealed for longer observation.

Methylene-blue reduction

Standardized methylene blue (British Drug Houses Ltd.) in concentrations of 0·1 and 0·01 % are mixed with suspension and sealed. Readings are made after 4 and 24 hr. at 37°.

Urease

Equal volumes of urea-buffer solution (urea 1 %, 0·0125 M buffer pH 6·0, phenol red 0·00025 %) and suspension are mixed. Sealed capillary tubes are incubated at 37° and read for alkali production after 4 and 24 hr.

TESTS ON AGAR COLUMNS

Gelatin hydrolysis

To detect gelatin hydrolysis Frazier (1926) flooded nutrient gelatin with acid mercuric chloride; this formed a thick white opalescence with the gelatin and left clear zones in areas of hydrolysis. Oakley, Warrack & Warren (1948) adapted the method to estimate gelatinases in bacterial toxins, using columns of 0·4 % gelatin and 1 % agar in water. For our microtest the columns were made up with 0·4 % gelatin and 0·5 % New Zealand agar. We found 0·5 % agar gave rather better results than 1 %; when the gelatin concentration was less than 0·4 % the opacity of the control tube was too faint. No difference was observed in the results for organisms grown on nutrient agar, serum agar, or 1 % glucose agar. The mercuric chloride diffuses slowly through the agar, and tests should be left on the bench for at least 30 min. before reading. The tubes were read against a blank gelatin-agar tube, and a positive reaction was indicated by a clear zone under the meniscus. (For a fuller description of the appearance of the tube, see Oakley *et al.* 1948.)

Cl. botulinum, *Cl. histolyticum*, *Cl. sporogenes*, *Cl. welchii*, *Ps. hydrophila*, *Staphylococcus aureus* and *Vibrio* spp. gave positive results at either 4 or 24 hr.; no positive reactions were obtained from organisms negative by the cultural method.

Method. Suspension is prepared from a 24 hr. culture grown on any suitable medium. For each test 0·04 ml. suspension is pipetted into each of two Durham's tubes containing 0·4 ml. gelatin-agar base. The tubes are placed in the air incubator at 37° and one is tested at 4, and the other at 24 hr. Acid mercuric chloride ($HgCl_2$, 15 g.; distilled water, 100 ml.; conc. HCl, 20 ml.) is added, and the tubes read after 30 min. at room temperature against a blank gelatin-agar tube treated in the same way.

Starch hydrolysis

The microtest for starch hydrolysis is based on the same principle as that for gelatin. The suspension is placed on a column of starch-agar and, at the end of the test period, Lugol's iodine is added to test for the remaining starch. The optimum composition of the column was found to be 0·2 % agar and 0·05 % potato starch.

Positive reactions were obtained in 2 hr. with the more active starch-hydrolysing organisms, but we decided to use 4 and 24 hr. as the test periods, so that the weaker reactors could be detected. Positive results were obtained with *Cl. sporogenes*, *Ps. hydrophila*, *Shigella dysenteriae*, *Sh. flexneri* and *Vibrio* spp.

Method. Suspension is prepared from a 24 hr. culture grown on any suitable medium. For each test 0·04 ml. suspension is pipetted into each of two Durham's tubes containing 0·4 ml. starch-agar base. The tubes are placed in the air incubator at 37° and tested at 4 and 24 hr. Lugol's iodine (2 % potassium iodide, 1 % iodine, in distilled water) is added and the tubes read after 30 min. at room temperature against a blank starch-agar tube treated in the same way.

Hydrogen sulphide

The production of H_2S from peptone media depends on the reduction of sulphur from sulphur-containing amino-acids or other sulphur compounds in the medium. We devised a micromethod for the detection of H_2S-production from cystine. Morse & Weaver (1950), in their microtest using 'thiopeptone', obtained satisfactory results with lead acetate paper. In the small volumes with which we were working the amounts of H_2S formed were insufficient to be detected by this method. We attempted to carry out the test with a strand of cotton impregnated with lead acetate inserted at the top of a capillary tube, but this was not sensitive enough. Finally we made the test at the top of a column of lead acetate agar, where the H_2S formed was detected by the development of a brown or black colour at the interface. The lead acetate agar base (LAAB) was prepared by adding 1 ml. of a 0·05 % solution of basic lead acetate (to which just enough HCl had been added to prevent precipitation) to 9 ml. 1 % New Zealand agar in water. LAAB was more sensitive than ferric ammonium citrate agar. The cystine was made up in 0·1 N-HCl and adjusted before use to pH 7·4–7·6 with dilute NaOH. Phosphate buffer was not used as it did not improve the sensitivity and, by reacting with the lead acetate, made the test more difficult to read. The addition of sodium thiosulphate had no effect on the speed or intensity of the reaction. The optimum pH was not critical; *Ps. hydrophila* gave positive reactions at pH values 6·0, 6·4, 6·8, 7·2, 7·6, 7·8 and 8, but the reactions were stronger and more rapid at pH 7·2–7·8.

With a heavy suspension of an H_2S-positive organism a black ring develops in 15–30 min. With most organisms the strength of the reaction and the rate at which it is produced depend on the concentration of the suspension. A 10^{12} organisms/ml. suspension of a 24 hr. culture of *Ps. hydrophila* produced a strong reaction in 30 min., but 10^{10} organisms/ml. gave no reaction in 24 hr.

Heavy suspensions of most of the organisms we tested gave positive results in 1–2 hr., but we left the tubes overnight so that weak reactions could be observed. This method is rather more sensitive than the cultural method for the detection of H_2S from peptone water. Positive results were obtained with *Bact. aerogenes, Bact. coli, Cl. histolyticum, Cl. sporogenes, Proteus morganii, Ps. hydrophila* and *Salmonella typhi.*

Method. Suspension is prepared from a 24 hr. culture grown on any suitable medium. To columns of 0·4 ml. LAAB in Durham's tubes are added 0·04 ml. cystine solution (0·1 % at pH 7·4) and 0·04 ml. suspension. The tubes are placed in the air incubator at 37° and read at intervals up to 24 hr.

OTHER TESTS

Nitrate reduction

The reduction of nitrate to nitrite was detected with dimethyl-α-naphthylamine (Wallace & Neave, 1927) and sulphanilic acid. The reaction was rapid with all the species tested; at 30 min. the results were consistent with the usual cultural method. We did not have any false negatives produced by reduction of nitrite, but these could be detected by zinc dust (ZoBell, 1932).

Method. Suspension, 0·04 ml., is mixed in 65 × 10 mm. tubes with 0·05 % $NaNO_3$, 0·06 ml.; phosphate buffer (0·025 M, pH 6·8), 0·04 ml. After 30 min. at 37° (water-bath) 0·06 ml. dimethyl-α-naphthylamine solution (6 ml./l. 5 N-acetic acid) and 0·06 ml. sulphanilic acid (8 g./l. 5 N-acetic acid) is added and read 5 min. later. A blank test on the nitrate and buffer is included with each batch of tests.

Acetylmethylcarbinol (*acetoin*)

Acetoin is formed from the breakdown of glucose by *Bact. aerogenes* and other bacterial species. Harden (1906) showed that it was the active substance in the Voges-Proskauer test; on the addition of strong potassium hydroxide the acetoin is oxidized to diacetyl which then condenses with substances in the medium containing a guanidine group to give a red colour. Acetoin can also be detected and estimated by oxidation to diacetyl and precipitation as the red nickel dimethylglyoxime (Lemoigne, 1920; Kluyver, Donker & Visser't Hooft, 1925), and estimated by iodine titration (Langlykke & Peterson, 1937). As the V.P. reaction is very sensitive and could easily be adapted to the micro-technique, we preferred a method derived from it. Glucose, creatine, and 0·025 M-phosphate buffer (pH 6·8) were used as the substrate; glucose at 10 % gave better results than 5 or 25 %. Creatine gave better results than 5 % peptone, arginine, or guanidine nitrate, and there was no significant difference between adding creatine with the glucose or adding it with the test reagents, 5 % ethanolic α-naphthol (Barritt, 1936) and 40 % KOH.

Medium and pH *value.* Suspensions of *Ps. hydrophila* from cultures grown 24 hr. on nutrient agar at pH 7·6 gave strong reactions after 1–2 hr. at 37° (water-bath), although *Bact. coli* and *Bact. aerogenes* were negative. After 24 hr. traces of acetoin could be detected with both *Bact. coli* and *Bact. aerogenes* suspensions. Strong reactions with *Bact. aerogenes* suspensions from nutrient agar were only obtained after 24 hr. in the presence of peptone, which may have allowed growth to take place. Suspensions of *Bact. aerogenes* from cultures grown 24 hr. on 1 % glucose agar or acid agar (nutrient agar at pH 4) gave strong reactions in 30 min., while *Bact. coli* suspensions from glucose or acid agar behaved as those from nutrient agar (Table 2). *Ps. hydrophila*, tested after 30 min., gave stronger reactions when grown on glucose agar (Table 2).

Table 2. *Acetoin production by suspensions from growth on different media*

Glucose 10 %, 0·02 ml.; creatine 0·2 %, 0·02 ml.; buffer 0·04 ml.; suspension, 0·04 ml.

	Suspension of culture								
	Bact. coli (NCTC 86)			*Bact. aerogenes* (NCTC 8348)			*Ps. hydrophila* (NCTC 7810)		
Time of test (hr.)	Medium on which culture had been grown								
	N.A.	G.A.	A.A.	N.A.	G.A.	A.A.	N.A.	G.A.	A.A.
	Score in test*								
½	0	0	0	0	6	4	4	8	0†
1	0	0	0	0	10	10	nt	nt	nt
4	1	0	1	0	10	10	nt	nt	nt
24	2	1	0	2	10	10	10	10	nt

N.A. = nutrient agar; G.A. = glucose agar; A.A. = acid agar.

* Numbers 0 to 10 represent relative scoring of strength of reaction; see Table 1.

† = no visible growth on acid agar; nt = not tested.

Silverman & Werkman (1941) showed that the *Bact. aerogenes* enzyme system responsible for acetoin production from pyruvic acid was only formed when the organism was grown on an acid medium, and that crude enzyme expressed from the bacteria had a pH optimum of about pH 5.

A pH curve (Sørensen's phosphate buffers 0·025 M or 0·06 M, pH 4·5–8·0) with suspensions of *Bact. aerogenes* and *Ps. hydrophila* grown on glucose agar, showed an optimum on the acid side of the range. After 2 hr. the breakdown of glucose had produced enough acid to overcome the effect of the 0·06 M buffer, and there was little difference in the results obtained in tubes with initial pH values between 4·5 and 7·2 (Table 3). We did not attempt to determine the pH optimum more accurately. For the microtests the greatest sensitivity was obtained by starting the reaction at a pH slightly higher than the optimum, and using a weak buffer. With no buffer, or if the reaction was started at a lower pH, the system became too acid. We therefore started our tests at pH 6·8 with 0·025 M buffer.

Time. The optimum incubation period depends on the sensitivity required. At 24 hr. the test is more sensitive than the usual cultural methods, and *Bact. coli* suspensions are weakly positive. Again, after the reagents are added and the tubes shaken to aerate the solutions, the rate at which the colour develops depends on the amount of acetoin present. With very small quantities the colour development may be delayed for 1–2 hr. For the detection of minimal quantities the suspension-glucose mixture should be left at 37° for 24 hr. and the test read up to 1 hr. after the reagents have been added. For routine use

Table 3. *Effect of* pH *value and buffer concentration on acetoin production by:* (P) Ps. hydrophila NCTC 7810 *and by* (B) Bact. aerogenes NCTC 8348

Glucose 10 %, 0·02 ml.; creatine 0·2 %, 0·02 ml.; buffer 0·04 ml.; suspension 0·04 ml.

		0·025 M phosphate buffer initial pH value†								0·06 M phosphate buffer initial pH value†							
Organism	Time of test (min.)	4·5	5·6	6·0	6·4	6·8	7·2	7·6	8·0	4·5	5·6	6·0	6·4	6·8	7·2	7·6	8·0
		Score in test*															
P	5	8	2	2	2	1	1	0	0	3	3	1	1	1	1	1	1
	15	6	6	4	6	5	2	3	4	4	4	4	3	3	2	2	2
	30	8	8	8	8	8	8	7	7	6	6	6	6	6	4	3	3
	60	10	10	10	10	10	10	10	10	8	8	10	10	10	10	10	10
	120	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B	5	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1	0
	15	4	4	4	3	2	2	1	1	2	2	1	0	1	0	0	0
	30	6	6	8	8	7	6	6	6	3	3	3	3	1	1	1	1
	60	8	8	9	9	9	9	9	9	6	6	6	6	3	2	1	1
	120	10	10	10	10	10	10	10	10	7	8	8	9	9	10	5	4

* Numbers 0–10 represent relative scoring of strength of reaction; see Table 1.

† Initial pH value; fermentation products cause a decrease in pH value during the experiment.

we adopted an incubation period of 1 hr. and read the tests 10 min. after the addition of the reagents. Under these conditions the test clearly distinguished between *Bact. coli* and *Bact. aerogenes* grown on glucose agar.

Method. Suspension is prepared from a 24 hr. culture grown on 1 % glucose agar, and 0·04 ml. suspension is added to a mixture of 10 % glucose, 0·02 ml.; 0·2 % creatine, 0·02 ml.; 0·025 M-phosphate buffer (pH 6·8), 0·04 ml.; in a 65 × 10 mm. tube. After incubation in a water-bath at 37° for 1 hr., 0·06 ml. 5 % ethanolic α-naphthol is added and the tubes shaken; 0·04 ml. 40 % KOH is then added, the tubes shaken again, returned to the water-bath for 10 min., shaken again and read.

Indole

The production of indole from peptone depends on the presence of tryptophan in the medium. When the suspension from a culture grown on nutrient agar is incubated with tryptophan, indole is formed fairly rapidly and can be detected by any of the indole test reagents. Kovac's reagent was preferred to Böhme's as the results were easier to read and only one solution was needed. Kovac's reagent was made up in pure *iso*amyl alcohol, which Arnold & Weaver (1948) found gave better results than the other alcohols tested. In a dark bottle the solution remained stable on the bench for several months; the colour remained a golden yellow and no false colorations appeared in the test.

Tryptophanase is only formed when the organism is grown in the presence of tryptophan and in the absence of glucose. Evans, Handley & Happold (1941) showed that non-viable suspensions of cultures grown with glucose or on tryptophan-free medium would develop tryptophanase when left in contact with tryptophan for a short time.

In the microtest for indole, indole-positive organisms were grown on nutrient or serum agar and suspensions gave positive results in $\frac{1}{2}$–1 hr. *Bact. coli* and *Bact. aerogenes* were grown on glucose agar for the acetoin test, and these suspensions were used to check the effect of growth on glucose on the other microtests. Only the indole reaction was affected and, as Evans *et al.* (1941) found, the inhibition of the tryptophanase activity by *Bact. coli* was overcome after the suspension had been in the presence of tryptophan for 1–3 hr. (Table 4). Similar results were obtained with *Ps. hydrophila* and *Pr. morganii.* It is, therefore, possible to carry out all the microtests on suspensions from glucose agar cultures if the duration of the indole test is prolonged.

Table 4. *Indole production by suspensions from growth on agar and glucose agar*

Tryptophan 0·1 %, 0·06 ml.; buffer, 0·04 ml.; suspension, 0·04 ml.

	Suspension of culture			
Time of test (hr.)	*Bact. coli* (NCTC 86)		*Bact. aerogenes* (NCTC 8348)	
	Medium on which culture had been grown			
	N.A.	G.A.	N.A.	G.A.
	Score in test*			
0·5	6	0	0	0
1	8	1	0	0
1·5	8	3	0	0
2	10	6	0	0
3	10	10	0	0
4	10	10	0	0

N.A. = nutrient agar; G.A. = glucose agar.

* Numbers 0–10 represent relative scoring of strength of reaction; see Table 1.

For very light suspensions and when older cultures are used, an incubation period of 4–6 hr. for the test may be necessary, but for our usual test we have taken an incubation period of 1 hr. when the organism is grown on nutrient or serum agar and 2–3 hr. when grown on glucose agar. Among the limited number of organisms tested no positive reactions were obtained for cultures negative by the cultural methods. *Bact. coli, Cl. bifermentans, Ps. hydrophila, Pr. morganii* gave positive results.

Method. Suspension is prepared from a culture grown on nutrient agar, serum agar, or 1 % glucose agar, and 0·04 ml. is added to a mixture of 0·1 % DL-tryptophan, 0·06 ml. and 0·025 M-phosphate buffer (pH 6·8), 0·04 ml. The tubes are incubated in the water-bath at 37° for 1 hr. (3 hr. when suspension is from a glucose medium) and 0·06 ml. Kovac's reagent (*p*-dimethylaminobenzaldehyde, 5 g.; *iso*amyl alcohol, 75 ml.; conc. HCl, 25 ml.) added. The tubes are shaken and read immediately.

DISCUSSION

The advantages of the methods described in this paper are simplicity of technique, clear-cut results, use of chemically-defined substrates instead of complex media, small amount of cells required to carry out a series of tests, rapidity and reproducibility. We have approached the bacterial cell as a system of preformed enzymes, and, using viable but non-multiplying suspensions, we tried to devise tests for the enzyme reactions used in bacterial characterization. With this in mind it is necessary to consider whether we want to assess the actual or the potential enzymic capacity of the cell. On nutrient agar at pH 7·6 *Bact. aerogenes* does not form the acetoin-producing enzyme, but it can be seen as a potential enzyme system because it is formed when the organism is grown on nutrient agar at pH 4–5, or on glucose agar. Again, the tryptophanase of *Bact. coli*, developed on nutrient agar, is inhibited when the organism is grown on a glucose medium. In this system the tryptophanase becomes active when the cells are in contact with tryptophan for about an hour. Therefore, if we want to realize the potentialities of the enzyme pattern, we may need to vary the conditions of our tests and to modify the medium on which the organism is grown. On the other hand, the ability to produce an enzyme on a particular medium may be of value in differentiating taxonomic groups; for example, the production of the acetoin enzyme by *Ps. hydrophila* on nutrient agar distinguishes it from *Bact. aerogenes*.

When the sensitivity of a test is increased, some organisms previously described as negative may be shown to give weakly positive reactions; this may arise either from an increase in the sensitivity of the test reagents, or from a change in the conditions of test. In this study only the H_2S, acetoin and fermentation tests gave greater sensitivity. For the H_2S test we devised a sensitive indicator, but with non-growing organisms, very heavy suspensions were needed. The detection of H_2S in culture media is made unsatisfactory by variation in media; in this laboratory we found that 0·1 % cystine added to nutrient broth made each batch more consistently useful for H_2S production as detected by lead acetate papers (ZoBell & Feltham, 1934), but our microtest, like that of Morse & Weaver (1950), is even more sensitive.

Fabrizio & Weaver (1951) showed that most coliforms are able to produce some acetoin; this can also be shown by using highly sensitive test reagents (Batty-Smith, 1941) on 10 to 14-day cultures in glucose phosphate peptone medium (unpublished observations). We may have to decide between detecting all the positive reactors up to the sensitivity limit of our tests or choosing conditions which will give consistency with the accepted cultural method.

So far these tests have been worked out and applied to only a limited number of strains. If experience shows that we cannot resolve the discrepancies between the tests on growing cultures and on washed suspensions we shall not be able to apply the microtests to ordinary diagnostic work. However, our methods show more clearly than growing cultures the fundamental enzymic make-up of bacteria, and may show unsuspected relationships between different species or genera. As a systematic study, a re-characterization of species on the basis of enzymic patterns may be just as important and profitable as antigenic analysis.

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Chaetomium brasiliensis Batista & Pontual; Nutritional Requirements for Growth and Fruiting

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SUMMARY: *Chaetomium brasiliensis* requires aneurin for growth, and calcium (strontium or barium), aneurin and a low nutrient level for the formation of ascospores. Decreasing suboptimal doses of calcium progressively decrease the number of fertile perithecia until none is formed, the average perithecial size is reduced, but not the total number of perithecia. Above the optimum level, calcium delays formation of perithecia and ascospores and, ultimately, spore germination; it has no favourable effect on vegetative growth. The fungus appears able to synthesize aneurin slowly after growth is complete.

Lilly & Barnett (1949) found that *Chaetomium convolutum* needs external supplies of both aneurin and biotin for growth and for ascospore formation. Apart from this, no essential vitamin requirements of Chaetomium species are known, although *C. cochliodes* fruits better in the presence of aneurin (Hawker, 1942) and that *C. globosum* fruits more quickly with added biotin (Buston & Basu, 1948). *C. globosum* and several other Chaetomium species, however, require calcium for the formation of mature perithecia and ascospores (Basu, 1951). In this paper it is shown that the recently discovered *C. brasiliensis* needs calcium for fruiting, and aneurin for both growth and fruiting.

MATERIALS AND METHODS

The test organism (IJMARI 117) was undescribed when first isolated by us from rotting jute fabric. According to the Commonwealth Mycological Institute, Kew, to which a subculture was submitted, it is identical with the fungus described by Batista & Pontual (1948) as *C. brasiliensis* and by Ames (1949) as *C. velutinum*. A subculture of the latter was kindly sent us by Dr Ames and we found it to be morphologically indistinguishable from our strain.

The organism was grown on malt agar slopes at 30° and stored in the refrigerator. The basal medium used was Czapek-Dox salt solution at pH 7·0 and contained: $NaNO_3$, 2 g.; K_2HPO_4, 0·75 g.; KH_2PO_4, 0·25 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; water, 1 l. The source of carbon was 0·2% glucose (w/v), unless otherwise mentioned. The medium was first prepared with half the required quantity of water and 5 ml. placed in 75 ml. American-made Pyrex conical flasks, which were cleaned before each test with hot chromic-sulphuric acid. After addition of supplements, the volume of medium in each flask was made to 10 ml. with distilled water. The flasks were sterilized at 5 lb./sq.in. for 10 min. Aneurin (as hydrochloride) was added before sterilization as a freshly prepared solution. Calcium was added as $CaCl_2$. Flasks were inoculated with a loopful of spore suspension and incubated at 30°.

As the organism produced numerous small, densely aggregated perithecia, counting perithecia was not possible. This was of little consequence because, unlike *C. globosum* and the other species previously examined, the number of perithecia, when these were formed, was always of the same order; differences were noticed in fertility and size of perithecia and in their terminal hairs. Whenever practicable cultures were duplicated.

RESULTS

Effect of aneurin on growth

On malt extract medium or on basal medium plus jute extract the fungus grew and fruited well, forming a deep smoky-black mycelium closely covered with blue-green perithecia having spirally curved terminal hairs; on basal medium alone growth did not proceed much beyond spore germination (Pl. 1, fig. 1), indicating a nutritional deficiency. Aneurin, biotin, riboflavin, pyridoxin, nicotinic acid and Ca pantothenate were added at concentrations of 0·1, 0·01, 0·001 μg./flask. The fungus responded only to aneurin, growth proceeding to completion with 0·01 μg. but not with 0·001 μg. aneurin/flask. At the lower concentration some sugar remained, the mycelium was white and no perithecia were formed; at the higher concentration sugar was exhausted, the mycelium turned black and its surface was extensively covered with greyish green perithecia which, however, showed more or less straight hairs, did not form either asci or ascospores and may be described as infertile. This state of growth (see Pl. 1, figs. 2 and 3), was not altered with increased doses of aneurin or combination of aneurin with any of the other substances mentioned above (at 0·2 μg./flask). A nutritionally adequate medium gives the same type of vegetative growth, but the perithecia, in addition to being fertile and covered with spirally curved hairs (Pl. 1, fig. 4), are darker and bigger.

The response of vegetative growth to increasing doses of aneurin was tested on basal medium which contained 3% (w/v) glucose and 0·2 M-phosphate buffer, and was adjusted to pH 6·0, the flasks being sterilized at 3 lb./sq.in. for 10 min. After growth the pH was still below 7·0. Buffer did not seem to affect growth or germination. Although glucose was still present, growth stopped in all flasks at the end of 20 days. The mycelia were washed once in distilled water, pressed lightly between filter-papers, dried at 110° for 8 hr. and, after cooling in a desiccator, weighed. Maximum growth, *c.* 75 mg. mycelium/flask, was obtained with 0·125 μg. aneurin HCl/flask, and half maximum growth at *c.* 0·04 μg. aneurin HCl/flask (see also Table 2). The mycelium was somewhat paler at low aneurin levels, darkening being absent when the amount of growth was very small. No distinct perithecia were formed. As infertile perithecia were formed on the 0·2% glucose medium, both with and without phosphate buffer, in the latter case the final pH was 8·2, it seems that the appearance of perithecia is dependent on the concentration of nutrients remaining in the medium rather than on its pH.

Requirements for fruiting

Calcium. Production of ascospores was induced by adding Ca to the medium, the results of one experiment being summarized in Table 1. Slight precipitates were observed in flasks containing 30 p.p.m. or more of Ca, but these disappeared as growth proceeded. At the end of the incubation period (30 days), no sugar was left in any flask. Perithecia were usually spherical, sometimes oval. Infertile perithecia were recognized from their smaller size, paler colour and deficiency in coiled hairs.

Table 1. *Effect of varying concentrations of calcium on perithecial development of* Chaetomium brasiliensis

(1 μg. aneurin HCl/flask present throughout.)

	Day of appearance of		Perithecia after 30 days	
Ca (p.p.m.)	Perithecia	Ascospores	Mean length (μ.)	Infertile (%)
0	9	np	62	100
0·1	9	np	82	100
0·4	9	11	92	64
1	9	11	87	48
3	9	11	98	25
5	9	11	100	20

Above Ca 5 p.p.m. the days of appearance of perithecia and ascospores respectively were: at 20 p.p.m. 9 and 11; at 50 p.p.m. 12, 14 and 14, 16 (duplicates); at 1000 p.p.m. 13, 14 and 15, 16 (duplicates).

np = none produced.

Ca increased the size of the perithecia and the number of fertile perithecia, the optimum Ca concentration being 3–5 p.p.m.; higher doses not significantly affecting these characteristics. No differences were detected in the degree of fertility, i.e. the number of spores per fertile perithecium. Above the optimum Ca range, other effects were noticed, the most important of which was delay in the maturation of perithecia. At Ca 30 p.p.m. and greater, the fungus began growth as white flakes and not as the normal mat but this difference soon vanished. At Ca 1000 p.p.m., germination was delayed by 2 days and the perithecia appeared as a scattered overgrowth and were probably fewer in number. At the concentrations tried, Ca did not affect the rate of growth or the total amount of growth (mycelium dry-weight) and had no effect without aneurin in the medium. With the formation of spores the colour of the perithecia changed to a deeper shade, from grey-green to blue-green, this colour change being an accurate indication of perithecial fertility.

In previous experiments with *C. globosum*, Ca was found to be replaceable by Sr and Ba, although particularly with Ba the number of mature perithecia formed was fewer (Basu, 1951). These elements also induced fruiting in *C. brasiliensis*, the effects being equal to that of Ca, when Sr and Ba were supplied at 10 p.p.m. (as nitrate and chloride respectively).

When Ca 10 p.p.m. was added, after completion of growth, to flasks initially without Ca and containing 1 μg. aneurin, there followed quickly a deepening of colour and formation of spores, the final result being the same as when Ca was added before inoculation. Thus Ca was concerned only with the process of fruiting and not with growth. The strain from Ames was also tested; the responses were the same as with the usual test organism.

Addition of a hot-water extract of jute to the basal medium caused good growth and fruiting. Mn, Fe, Co, Ni, Sn, and Pb, found in the ash of jute extract by Basu (1951), were tested at 1 and 10 p.p.m. in the medium but no effect on growth or fruiting was observed; Co and Ni were inhibitory to growth.

Table 2. *Effect of aneurin on fruiting of* Chaetomium brasiliensis

(Ca 10 p.p.m. present throughout.)

Aneurin HCl (μg./flask)	Day of appearance of Perithecia	Day of appearance of Ascospores
0·001	np	np
0·003	np	np
0·006	np	np
0·010	26	30
0·020	20	23
0·030	18	21
0·040	11	17
0·050	9	11
0·075	9	11
0·100	9	11

Glucose remaining after 30 days' incubation were 9·1, 6·1 and $<2·0$ mg./flask with 0·001, 0·003 and 0·006 μg. (or higher) aneurin HCl/flask.

np = none produced.

When Whatman no. 5 filter-paper was used as the source of carbon instead of glucose and aneurin was supplied, *C. brasiliensis*, like *C. globosum*, formed many fertile perithecia in 7 days without added Ca. Fewer fertile perithecia formed by *C. brasiliensis* with Whatman no. 30 and no. 44 filter-papers, which have progressively smaller ash contents, only a few spores being formed with no. 44, although they appeared equally quickly with each filter-paper tried. That *C. globosum* fruited equally well on all these filter-papers is probably related to the fact that, unlike *C. brasiliensis*, it sporulates to some extent on a medium with a very low initial glucose content. The reaction of *C. brasiliensis* to the different filter-papers was reproduced when equivalent quantities of their ash were added to the usual glucose medium. Ordinary macro-chemical analysis showed Ca, but not Sr or Ba, in the ash of no. 5 filter-paper. Cellulose decomposition of filter-paper, determined by weighing the residue of no. 5 filter-paper left after 13 days' growth, was the same with both species, with or without Ca 10 p.p.m. added to the medium, which is consistent with the observation that Ca does not affect vegetative growth.

Aneurin. In Table 2 are given the results of an experiment designed to see whether aneurin was necessary for fruiting, apart from growth. Germination and rate of growth of mycelium did not appear to vary with the dosage of aneurin. Fruiting was progressively delayed below 0·05 μg./flask and at 0·006 μg./flask no perithecia were formed in 30 days, although growth had reached completion. It seems that a much higher concentration of aneurin is needed for fruiting than for growth and that the fungus can slowly synthesize the extra amount after growth is complete, when a little more aneurin than needed to reach this stage is originally present. The average size of perithecia and the percentage of infertile perithecia at 45 days did not differ significantly between flasks and were of the same order as obtained under optimum conditions (Table 1). The same applies to the total number of perithecia, except that at 0·01 μg. aneurin/flask the number was smaller.

Sugar concentration. Apart from adequate amounts of Ca and aneurin, another requirement for fruiting is that the concentration of free sugar in the medium must be very low; this was indicated by the quicker fruiting of the fungus on filter-paper. With increasing amounts of glucose in the medium fruiting was correspondingly delayed, at the following initial concentrations of glucose (%) the days of appearance of perithecia and ascospores respectively were: 0·2%, 8, 11; 0·3%, 10, 14; 0·3%, 13–15, 15–18; 0·5%, 15–18, 20–21; 0·6%, 21, 23. In every case less than 2 mg. glucose were left when ascospores were first seen. In another experiment, with 2% glucose, 1 μg. aneurin, and 20, 50 or 100 p.p.m. Ca in the medium fruiting did not take place even when only 18 mg. sugar were left after growth. Even immature perithecia are not formed, either with or without Ca in the medium, unless the sugar content approaches exhaustion.

DISCUSSION

A low nutrient level favours fruiting in several other species of *Chaetomium* (Basu & Bose, 1950), *C. convolutum* (Lilly & Barnett, 1949) and in some other Ascomycetes (Asthana & Hawker, 1936; Hawker & Chaudhuri, 1946). In general, environmental conditions which restrict growth favour reproduction, particularly the formation of sexual spores.

In its aneurin requirement for growth, *C. brasiliensis* resembles *C. convolutum* which is the only other Chaetomium species shown to have a specific vitamin requirement. Lilly & Barnett (1949), examining the effect of aneurin on *C. convolutum*, found that at a given nutrient concentration, increasing doses of aneurin successively initiated vegetative growth, formation of perithecia and of ascospores; within certain limits, the number of mature perithecia and ascospores increased. With *C. brasiliensis* more aneurin is needed for the formation of perithecia than for complete vegetative growth, and when enough calcium is present fruiting always follows the appearance of perithecia. The only other noticeable effect of aneurin on this species was in the acceleration of the appearance of perithecia and ascospores. *C. brasiliensis* does not require an external supply of biotin, which has the same effect as aneurin on *C. convolutum*.

Instances of the stimulation of spore formation by calcium are rare. Brian & Hemming (1950) found that conidia formation by the imperfect fungus *Trichoderma viride* was stimulated by 0·01 to 0·1% calcium chloride. Stimulation of sexual spore formation by calcium, strontium or barium is known only in other species of *Chaetomium*, in particular *C. globosum* (Basu, 1951). The replaceability of calcium by strontium or barium is even more marked in *C. brasiliensis* than in *C. globosum*, but manganese, which engendered a few fertile perithecia in the latter, had no effect on the former. Similarity with *C. globosum* is also observed in that calcium has no effect on vegetative growth and both species react to calcium even when added after complete growth. However, from a consideration of the two strains of *C. brasiliensis* and the three strains of *C. globosum* tested, a rather fundamental point of difference is noted between the two species, that while in *C. brasiliensis* calcium induces only the formation of ascospores in the large number of infertile perithecia which develop even without the metal, in *C. globosum* the transition is from a few or no perithecia, infertile or fertile, to a large number of fertile perithecia.

I wish to thank the Research Director, Indian Jute Mills Association Research Institute, for permission to publish these results.

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EXPLANATION OF PLATE

Fig. 1. Culture of *C. brasiliensis* after 14 days on the basal medium. × 1·4.

Fig. 2. Culture of *C. brasiliensis* after 14 days on the basal medium plus 1 μg. aneurin, showing densely aggregated infertile perithecia. × 1·4.

Fig. 3. Crushed perithecium of *C. brasiliensis* after 20 days on basal medium plus 1 μg. aneurin, showing absence of asci, ascospores or curved terminal hairs. × 250.

Fig. 4. Crushed perithecium of *C. brasiliensis* after 14 days on basal medium plus 1 μg. aneurin and 5 p.p.m. calcium, showing cylindrical asci, ascospores, and curved terminal hairs. × 250.

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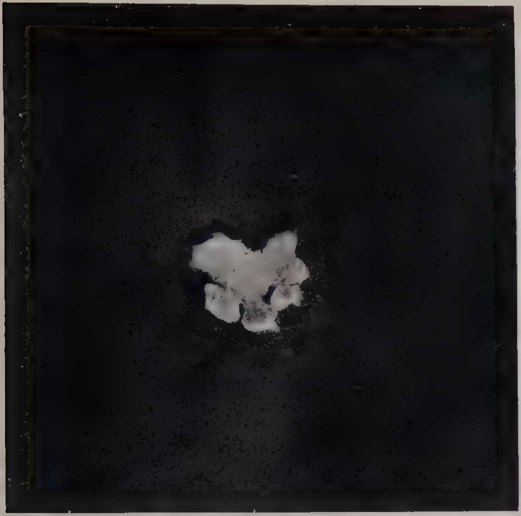

Fig. 1

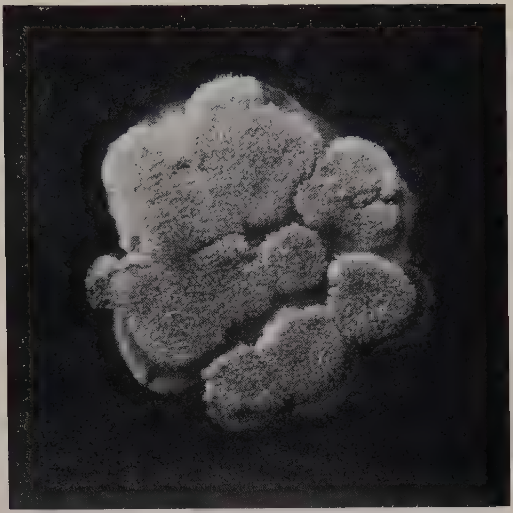

Fig. 2

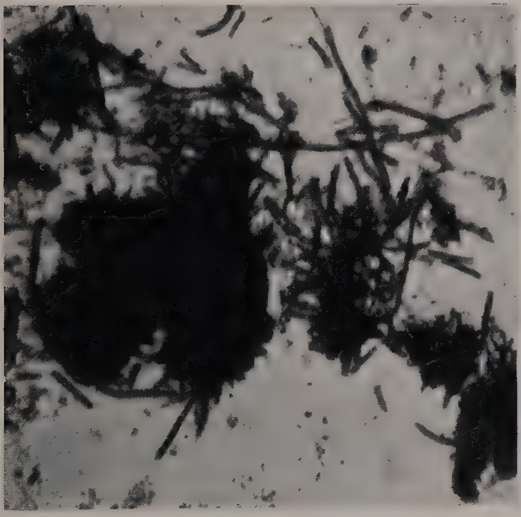

Fig. 3

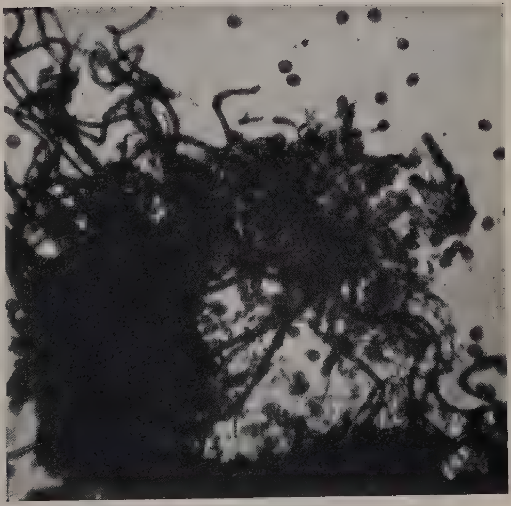

Fig. 4

S. N. Basu—Nutrition of *Chaetomium brasiliensis*. Plate 1

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The Micrococci in a Marine Environment

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SUMMARY: Micrococci forming part of the marine flora, are especially important in fish spoilage. They are common in estuarine mud but rare in sea water and in marine mud of the Australian continental shelf. Non-pigmented and orange strains are most abundant; lemon-yellow strains are found in the early stage of spoilage of teleost fish, but are rare in mud. About 10% of the cocci form packets, and these are commonest in mud. Nearly all marine micrococci are saccharolytic, hydrolyse urea, and attack asparagine, aspartic acid and alanine. The classification and taxonomic position of these cocci are discussed.

Species of *Staphylococcus* (*Micrococcus*) are normal inhabitants of the slime of teleost fish (Wood, 1940), and of elasmobranchs (Wood, 1950). They are found in the early stages of teleost spoilage and in all stages of elasmobranch spoilage; in this they may play a part as they often attack urea, peptone, asparagine, aspartic acid, and alanine. At times they are abundant in estuarine and marine muds, but they are not common in sea water or on submerged surfaces.

The fish examined were all demersal and it is probable that the organisms they carried came from the mud.

MATERIALS AND METHODS

Nearly 600 cultures were examined from various marine sources: fish muscle, the surface of living fish, sea water, estuarine and marine muds.

The methods used were those recommended in the Manual of Methods published by the Society of American Bacteriologists. Reactions studied were: Gram reaction (Hucker's modification); growth on agar and on potato; action on milk; nitrate reduction; hydrolysis of starch and gelatin; H_2S-formation; production of NH_3 from urea, peptone, asparagine, aspartic acid, glycine and alanine; catalase production; and the fermentation of glucose, lactose, sucrose, maltose, mannitol, galactose, fructose, glycerol, salicin, inulin, raffinose and xylose.

RESULTS

All strains grew on sea-water or tap-water agar and had an optimal growth temperature of 20–30°; a few strains did not grow at 37° but most were heat tolerant and grew above 45°. They were nearly always Gram-positive.

The results (Table 1) suggest that the strains are of the usual mesophilic type, and cannot be regarded as specific to the marine environment from which they came. There was no definite grouping of the strains, and, apart from fermentation, there seemed to be a random distribution of all characters. The grouping used in Tables 1 and 2 is explained later in this paper.

Table 1. *Reactions of micrococci from marine sources*

	Species-group					Species-group			
	Staph. candidus	*Staph. aurantiacus*	*Staph. luteus-varians*	*Staph. roseus*	*Staph. flavus*	*Sar. candida*	*Sar. aurantiaca*	*Sar. lutea-citrea*	*Sar. schaudinii*
No. of strains	283	99	124	8	4	18	14	11	4
	Percentage of strains giving positive reactions								
Gelatin liquefied	24	63	35	0	100	5	30	0	25
Reaction of milk:									
Acid	40	15	13	25	75	50	23	0	50
Alkaline	28	44	65	37	0	18	77	100	0
Unchanged	32	39	22	38	25	34	0	0	50
Nitrates reduced	43	32	48	50	75	44	30	27	0
Acid from:									
Glucose	88	77	50	62	100	83	68	63	100
Lactose	65	90	33	25	50	60	20	18	75
Sucrose	93	55	33	62	100	72	42	54	100
Maltose	88	77	33	50	100	80	30	45	75
Mannitol	77	77	33	50	50	72	71	36	100
Galactose	65	90	33	50	50	21	36	27	75
Fructose	80	77	33	75	50	60	64	27	100

Less than 1 % of the staphylococci failed to ferment at least one sugar, but in the packet-forming cocci (sarcina) the proportion of non-fermenters was higher. Glucose, sucrose, maltose and fructose were most often attacked, but strains which failed to ferment glucose, yet fermented mannitol, fructose or maltose, were numerous, possibly because seaweeds contain galactans and mannuronic acid. Uniformity in reactions was only found when a small number of strains was tested, and one feels that species descriptions based on a few strains have little validity.

The correlation between the eventual grouping of the strains and their sources is shown in Table 2. The non-pigmented forms (*Staph. candidus*) were widely distributed, but the lemon-yellow (*Staph. luteus*, *Staph. varians*) and

Table 2. *Source of marine cocci*

(Number of strains.)

Species-group	Teleosts	Elasmobranchs	Sea-water	Estuarine mud	Glass plates	Marine mud
Staph. candidus	197	15	11	60	0	1
Staph. aurantiacus	89	4	0	5	0	2
Staph. luteus	43	0	0	0	0	0
Staph. varians	75	1	0	3	0	0
Intermediate	0	2	1	1	0	0
Staph. flavus	0	4	0	4	0	0
Staph. roseus	0	1	3	4	0	0
Sar. candida	1	0	0	18	–	2
Sar. schaudinii	0	0	0	4	–	0
Sar. ureae	0	0	1	0	–	0
Sar. aurantiaca	1	4	1	9	–	1
Sar. lutea	1	4	0	6	–	0
Sar. citrea	0	0	0	1	–	0

orange (*Staph. aurantiacus*) forms were found chiefly on or in fish. In a marine environment the orange and white strains formed 73 % of the cocci, but in fish muscle only 60 %. Although coccoid forms were seen on glass plates immersed in the sea, no cultures of cocci were isolated from them. It is possible that coccoid forms of *Corynebacterium* species were seen.

Taxonomy

There was sometimes difficulty in deciding whether a strain was a staphylococcus or a sarcina, but several strains of large cocci in packets were undoubtedly sarcina. Bridging forms between bacterial genera are so common that an arbitrary separation is often inevitable.

One difficulty in classifying micrococci is to decide whether a difference in growth optimal temperature (25–37°) is sufficient to delimit two species-groups. Some marine strains grow at 37° but do not produce coagulase; they form a transition group between the pathogenic staphylococci and the non-pathogenic micrococci. Pathogenicity is not a valid character in the separation of groups, as it is purely relative.

In Hucker's classification (1924*a–d*, 1926, 1928) gelatin liquefaction and nitrate reduction are used as distinguishing characters, but my results agree with those of Abd-el-Malek & Gibson (1948) who found that these characters had only a limited value. In my series the ability of individual strains to reduce nitrates or to liquefy gelatin were not stable characteristics.

The pigmentation of the yellow-green forms seems to be constant; only one white variant was found. However, in the orange group certain strains regularly formed non-pigmented sectors. These non-pigmented forms bred true but the pigmented continued to produce non-pigmented sectors in some colonies but not in others, a phenomenon suggestive of dominant and recessive characters. Hoffstadt & Youmans (1938) produced stable white variants from *Staph. aureus* which were serologically identical with the parent culture. However, Byatt, Jann & Salle (1948), by using an extract of *Staph. aureus*, induced a yellow form of *Staph. albus*. If pigmentation is used as a differential criterion this type of variant must be considered; it is independent of the medium and has to be distinguished from the transient variation due to differences in medium.

Many strains from marine sources appeared to be truly non-pigmented, but unless the history of each strain is known it is not possible to distinguish the true non-pigmented strain from the non-pigmented variant. There seems to be a reasonable case for separating the orange-yellow from the non-pigmented forms. Eight strains producing a pink pigment confirmed Hucker's findings that pigmentation is a constant character in this group.

Gibson (personal communication) considers that pigmentation is of little importance as a differential character, but I agree with Hucker that it has considerable value in the classification of micrococci. Supporting this view is the absence of yellow or orange strains in sea water (Table 2) and the association of pink cocci with high salt concentrations. Cocci producing pink colonies are rare in an oligo- or meso-haline environment; none occurred in teleost fish flesh and only one on the skin of an elasmobranch. I consider that pigment has an ecological significance in the cocci of the soil-estuary-marine system. There is a remarkable, and possibly significant, similarity between the pigment range of the micrococci and the corynebacteria; comparative studies of pigmentation might help to clarify their relationships. Of the marine strains examined, more than half were non-pigmented, and about a fifth produced an orange pigment. These could be placed in Abd-el-Malek & Gibson's group I (the staphylococcus group); none fell into subgroup IA, and the majority were in subgroups IC and ID, but the separation of these subgroups. seemed arbitrary. Four non-fermenting strains fell into group II, and about a quarter into group III (the luteus-varians group). The yellow and pink strains form two additional groups; they are constant in colony character and apparently homogeneous in their reactions.

The classification suggested is based on Abd-el-Malek & Gibson's (1948) and is as follows:

Group I, subgroups A and B (staphylococcus group 1).
    Staph. albus group.
    Staph. aureus group.
        subgroups C and D (staphylococcus group 2).
    Staph. candidus group.
    Staph. aurantiacus group.
Group II, an intermediate group, represented in this series by only four strains and considered to be part of a transition series.
Group III, subgroup A, *Staph.* (*Micrococcus*) *luteus* group.
        subgroup B, *Staph.* (*M.*) *varians* group.

These two subgroups are composed of purely saprophytic forms which are mostly heat tolerant (45° or higher).

Group IV, *Staph.* (*M.*) *flavus* group.

Represented by eight strains in this series (only four tested for biochemical characters); pigmentation was bright yellow and distinct in shade from other groups.

Group V, *Staph.* (*M.*) *roseus* group.

The eight cultures supported Hucker's statements that the group is distinct.

The specific names used in this paper indicate species-groups in the sense of Abd-el-Malek & Gibson rather than species in the sense of *Bergey's Manual* (1948). It is difficult to decide what names should be used in this genus; by modern standards the original descriptions of species are inadequate, but other authors using these names have gradually added to the description. If we are to accept only six or seven species of *Staphylococcus* it would be best to retain the oldest available names, although the original descriptions suggest that they may be *nomina nuda*. The only alternative is a new set of names. We should remember that Cohn's conception was of a species-group rather than of a Linnean species. The use of species-groups instead of numbered or lettered groups is preferable because binomials are familiar to biologists. Microbiologists now realize the difficulty of separating phylogenetic species in the lower order of plants, and the species-group has a recognized place in classification.

I prefer *Staph. albus* Rosenbach 1884 and *Staph. aureus* Rosenbach 1884 to *Staph.* (*M.*) *pyogenes* vars. *albus* and *aureus*, as I consider that the white and orange forms are specifically distinct. I have only observed loss of pigmentation on plates where white and orange forms co-existed, and have never seen it in orange forms isolated in pure culture.

Similarly, with the specific names *Staph.* (*M.*) *candidus* Cohn and *Staph.* (*M.*) *aurantiacus* (Schroeter) Cohn; both descriptions developed by *Bergey's Manual* (1948) appear to fit organisms isolated from marine sources.

The species names *Staph.* (*M.*) *luteus* (Schroeter) Cohn and *Staph.* (*M.*) *varians* Migula 1900 are used for the marine forms which appear to correspond to Abd-el-Malek & Gibson's group III, and they agree with the descriptions in *Bergey's Manual*. The description of *Staph.* (*M.*) *flavus* Trevisan 1889 seems to fit the few strains of group IV isolated from marine sources. If the pink forms are grouped as a species, as I think they should be, the name *Staph.* (*M.*) *roseus* Flügge 1886 has priority.

Marine Sarcina *strains*

The reactions of the packet-forming cocci (sarcina) examined are shown in Table 1, and their sources in Table 2. Pigmentation seemed to be the main criterion for classification; orange, lemon-yellow, and non-pigmented forms were found. The non-pigmented forms were either motile or non-motile, or motile with spores. One lemon-yellow strain was motile but in other characters resembled *Sarcina lutea*. As in the micrococci, species-groups rather than species are recognizable. The non-pigmented forms are not described in *Bergey's Manual*, but they appear to fit the descriptions of *Sar. candida* Lindner and *Sar. schaudinii* (Wolff) Pribram. The orange strains were *Sar. aurantiaca*, and the spore-forming species described as *Sar. ureae* (Beijerinck) Lohnis by Wood (1946) should be named *Sporosarcina ureae* Beijerinck.

Species of *Sarcina* are commoner in sea water and mud than on fishes (Table 2); this may be a significant ecological difference between *Sarcina* and *Staphylococcus*.

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Heritable and Non-heritable Loss of Ability by *Aerobacter aerogenes* to Grow Adaptively on Single Carbon Sources

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SUMMARY: Mutants of *Aerobacter aerogenes* incapable of utilizing citrate (C−) were discovered in glucose cultures by a modification of the mutant-concentrating penicillin technique. These C− mutants utilize glucose as efficiently as does the wild-type (C+), despite their inability to utilize a wide range of other metabolites as carbon sources for growth, including compounds that would be oxidized if glucose were aerobically dissimilated via the Krebs cycle. The compounds that the C− mutants cannot utilize are citrate, α-ketoglutarate, glutamate, succinate, fumarate, aspartate and acetate, which are all substrates to which glucose-grown C+ cells slowly adapt. A non-genetic loss of ability to grow in media containing these substrates also occurs in glucose-grown populations under certain conditions. The C− mutant can revert to the prototrophic condition directly, or indirectly by first mutating to a stable state (C− F+) in which fumarate and aspartate can be utilized whereas the other compounds cannot.

The ability of the coliform group of bacteria to utilize the so-called 'Krebs cycle compounds' is of considerable genetic, evolutionary and biochemical interest. These bacteria have long been regarded as a closely related group because of many morphological and physiological similarities (*Bergey's Manual*, 1948). Certain specific biochemical characters are, however, not uniformly distributed among the species in this group. Mutation is probably the process by which these differences have come about. In some strains of *Escherichia coli* (*Bacterium coli*), for example, the inability to utilize citrate, which is one of the characters used to define the species, has been reported to be affected by mutation (Parr, 1938).

Many coliform organisms which utilize Krebs cycle metabolites do so adaptively. For example *Aerobacter aerogenes*, after growth in a defined medium containing glucose as sole carbon source, shows adaptive utilization of acetate, succinate, fumarate and α-ketoglutarate (Baskett & Hinshelwood, 1949; Ajl, 1950). In the present study it was found that the ability to adapt to these compounds can undergo a stable mutation, which in no way affects the ability to utilize glucose. In addition, it was found that the ability to adapt can be lost temporarily as a result of ultraviolet irradiation or exposure to conditions which prevail in the stationary phase of certain glucose cultures.

In bacteria the relation of glucose metabolism to the dissimilation of the di- and tri-carboxylic acids remains obscure. Doubt has been raised as to whether the Krebs cycle operates in the aerobic dissimilation of glucose by coliform bacteria because of experiments in which the oxidation of various di-carboxylic acids was blocked by specific inhibitors without affecting the oxidation of glucose, e.g. succinate oxidation by malonate (Lenti, 1946; Aubel, Rosenberg & Szulmajster, 1950); α-ketoglutarate oxidation by arsenious oxide (Ajl, 1950); fumarate oxidation by cyclohexanol (Ajl, 1950). Of particular significance is the finding that fluoroacetate has no effect on the growth of *A. aerogenes* in glucose medium (Dagley, Dawes & Morrison, 1949) although it blocks the Krebs cycle in brain and kidney tissues (Buffa, Coxon, Lièbecq & Peters, 1949). The question cannot be considered closed, so far as the coliform bacteria are concerned, because of recent findings that aconitase, isocitric dehydrogenase, and the enzyme catalysing the synthesis of citrate from oxaloacetate and acetate, can be demonstrated in extracts prepared from *Esch. coli* (Novelli & Lipmann, 1950; Dr J. R. Stern, personal communication).

Thus, enzyme investigations have been successful in isolating from *Esch. coli* active forms of the enzymes involved in the Krebs cycle, while inhibitor studies suggest that the Krebs cycle does not function in the aerobic dissimilation of glucose by this organism. Possibly the Krebs cycle constitutes a potentially active system in coliform organisms but does not operate during glucose dissimilation. Consistent with such a view are the results of a comparison of the physiology of the C+ strain with a C− mutant (incapable of utilizing citrate) of *A. aerogenes* to be described in this paper.

METHODS

The C+ (wild-type) strain of *A. aerogenes* used was strain no. 211 from the American Type Culture Collection. The S− and S+ strains of *Esch. coli* K12 (strains incapable and capable of utilizing succinate, respectively) were generously furnished by Dr T. C. Nelson. Stock cultures were maintained in the refrigerator on nutrient agar slopes, and were transferred every 3 months to fresh media.

Liquid cultures were prepared as follows. A small amount of the slope growth was suspended in physiological saline, and a suitable volume of this suspension was inoculated into the medium from which the experimental inocula were to be taken. When the culture was in the log phase of growth, it was placed in a sterile centrifuge tube, centrifuged, the supernatant decanted and the bacterial pellet resuspended in a volume of saline equal to that of the culture used. Measured volumes of this bacterial suspension were then inoculated into the test media. Unless otherwise indicated, 6 ml. volumes of test media contained in 16 × 150 mm. Pyrex test-tubes were used.

Test media. The media used included: (1) Difco nutrient broth (0·3 % meat extract + 0·5 % peptone); (2) a defined medium, the inorganic salt component of which consisted of 5 g. NH_4Cl; 1 g. NH_4NO_3; 2 g. Na_2SO_4; 0·1 g. $MgSO_4 . 7H_2O$; *c.* 1 mg. $CaCl_2$; 3 g. K_2HPO_4; 1 g. KH_2PO_4; 1 ml. trace elements solution (Ryan, 1950); made up to 1 l. in distilled water. The carbon source in the defined medium was usually 0·5 % (w/v) glucose. When a different carbon source was used, its concentration was equivalent, on a carbon-for-carbon basis, with 0·0277 M (0·5 %) glucose. Such a concentration is referred to in the text as the standard concentration of the substrate. With the exception of D-glucose, the DL-forms of optically active carbon sources were used. The defined medium was adjusted with NaOH so that its pH value after sterilization was 6·8–7·0. This pH measurement, as well as all others described below, was made with a Beckman glass-electrode pH meter. Washed agar (Ryan, 1950) was used at 1·5–2·0% (w/v) for preparing solid media. Media were sterilized by autoclaving at 15 lb./sq.in. pressure for 10 min. When solid media were used, the carbon source + agar component was sterilized separately from the mineral component. Reagent grade chemicals were used.

Cultivation. All cultures were incubated at 37° in a constant-temperature room, where all measurements were made. Flask cultures, when used, were aerated in a modified shaking tank of a Warburg apparatus. Test-tube cultures were aerated by means of a roller device, which, according to Prof. F. J. Ryan (personal communication), assures optimal aeration.

Measurement of growth. Growth was measured turbidimetrically in a Klett-Summerson colorimeter. The relation between number of viable bacteria/ml. (determined by plating) and the colorimeter readings was found to be linear over the range of 0–120 colorimeter units. At 150 units the deviation from a linear relationship was about -5%; above 150 units the deviation increased until at 350 Klett units the deviation was about -15 to -20%. Over the linear range each colorimeter unit represented about 5×10^6 bacteria/ml. In the figures illustrating this paper, the optical densities of the cultures in Klett units are plotted on a logarithmic ordinate scale.

Colony counts. The determinations of the number of cells present in a given culture that were capable of utilizing both glucose and some other substrate as carbon sources were made by appropriately diluting a culture sample and plating in two sets of defined media: (1) glucose agar (G); (2) agar containing the other substrate (X) as the carbon source. After suitable incubation counts were made of plates containing from 40 to 400 colonies/plate, and an average was taken of the counts of at least two plates. Since the difference between the number of colonies on any two plates containing the same carbon source never exceeded 20%, a ratio between the two averages (G:X) greater than 1·5, or less than 0·67, is significant.

Ultraviolet irradiation was accomplished by placing *c.* 5 ml. of a saline suspension of bacteria in a Petri plate and exposing the bacteria to the irradiation of a Westinghouse 'Sterilamp', which delivers approximately 85% of its radiant energy at 2537 A.

RESULTS

Characteristics of growth of wild-type (C+) *in defined media*

Growth in media containing glucose as sole carbon source. Table 1 summarizes the effects of glucose concentration and degree of aeration of medium upon (1) growth rate (expressed as the mean generation time during the log phase of growth), (2) final level of growth, (3) limiting factor of growth.

Addition of glucose to cultures which had ceased to grow revealed that glucose exhaustion limited growth when the initial glucose concentration was 0·05%. However, when the initial glucose concentration was 0·5%, the addition of glucose during the stationary phase produced no growth response, for

Table 1. *Influence of glucose concentration and degree of aeration upon growth of* C+ (*wild-type*) A. aerogenes *in defined medium*

Growth of tube cultures at 37° was measured turbidimetrically in a Klett-Summerson colorimeter. Rate of growth is given as the mean generation time (or time for doubling of the population during the log phase). pH values determined by Beckman glass electrode pH meter. Limiting factors of growth were determined by additions of glucose at end of growth and of buffer at time of inoculation (see Table 2). The G:C value given is the ratio of the count of colonies formed in glucose agar to the count of those formed in citrate agar as a result of plating an appropriately diluted sample of the culture at time indicated.

Glucose (%)	Aeration	Mean generation time (log phase; min.)	Ratio G : C (log phase)	Final amount of growth (Klett units)	Final pH value	Limiting factor of growth	Ratio G : C (15 hr. stationary phase)
0·05	Unaerated	43	0·9	75	6·6	Glucose-exhaustion	1·1
0·05	Aerated	32	0·9	110	6·7	Glucose-exhaustion	1·0
0·5	Unaerated	43	1·0	154	5·6	Low pH value	4·6
0·5	Aerated	32	1·0	310	5·8	Low pH value	1·1

Table 2. *Influence of buffer capacity of medium upon final amount of growth of* A. aerogenes

Growth of C+ (wild-type) tube cultures containing 0·5 % glucose in unaerated defined medium at 37° was measured turbidimetrically. Buffer strengths in the medium are represented as the standard phosphate concentration (1 ×) or two (2 ×), four (4 ×) or eight (8 ×) times this concentration.

Buffer strength in medium	1×				2×		4×		8×	
Proportion of dibasic to monobasic phosphate	3 : 1	2 : 1	1 : 1	1 : 3	3 : 1	2 : 1	3 : 1	1 : 1	3 : 1	1 : 3
Final amount of growth (Klett units)	173	161	131	66	246	231	292	236	331	242
Initial pH value of medium (after autoclaving)	6·8	6·6	6·4	6·1	6·9	6·7	6·9	6·5	6·9	6·1

in these cultures the acid pH value was the limiting factor. This is shown in Table 2, which gives typical results obtained in unaerated 0·5 % glucose media. An increase of the buffer concentration of the medium, without change of its initial pH value, greatly increased the amount of growth, while a decrease of the initial pH value of the medium at any buffer concentration significantly decreased this amount of growth. Phosphate was not the only buffer that could achieve this effect. The presence of citrate in unaerated 0·5 % glucose media increased the final crop of the C− mutant strain, which, as will be seen below, cannot utilize citrate as a carbon source for growth, thus presumably leaving this substance intact to act as a buffer. Our conclusion that decreased pH values limited the growth of cultures of *A. aerogenes* under conditions where glucose was non-limiting, is in disagreement with that of Dagley, Dawes & Morrison (1951). From the observation that the population density of unaerated cultures of *A. aerogenes* grown in the presence of excess glucose could be considerably increased by subsequent aeration, these authors concluded that low pH values did not adversely affect the cells. However, it is possible that aeration permits the resumption of growth precisely because the cells can more fully utilize some of the organic acids accumulated through glucose breakdown.

For reasons not yet understood, a serially-transferred aerated glucose culture of *A. aerogenes* occasionally changes from the faster growth rate (mean generation time = 32 min.) typical of aerated cultures to the slower growth rate (mean generation time = 43 min.) characteristic of unaerated cultures. Prolonged serial transfer often results, however, in a reversion to the faster growth rate.

Adaptation of glucose-grown cells to other carbon sources. Cells grown in medium containing glucose as carbon source (to be referred to as *glucose-grown cells*) were able to grow in media containing any one of the following compounds as sole carbon source: glucose, glycerol, pyruvate, lactate, alanine, malate, aspartate, fumarate, succinate, acetate, α-ketoglutarate, glutamate, citrate (but not formate). As Baskett & Hinshelwood (1949) observed, with another strain of *A. aerogenes*, glucose-grown cells must adapt to the utilization of some of these compounds. I always observed an appreciable lag period (greater than 8 hr.) following the transfer of *c.* 10^6 to 10^7 washed, log-phase, glucose-grown cells into media containing α-ketoglutarate, succinate, fumarate, acetate, glutamate or aspartate as carbon source. A brief lag (up to 2 hr.) was observed with glucose, glycerol, pyruvate, malate or alanine. With citrate as sole carbon source, very often this brief lag was noted, but occasionally a longer lag period of 3–4 hr. was observed. Cells which have become adapted to one of the former group of compounds, as indicated by the absence of lag upon reinoculation into medium containing that compound as sole carbon source, must readapt to the utilization of that compound after transfer through glucose medium. Thus, of the substrates investigated, two classes may be distinguished: those which require a slow adaptive process before utilization by glucose-grown cells, and those which do not. As will be shown, the substrates in the former category are apparently related to each other by virtue of a particular biochemical system existing in *A. aerogenes* in which a metabolic block imposed by mutation leads to the loss of ability to utilize these compounds.

Diauxic growth in media containing glucose + another carbon source. When glucose-grown cells are inoculated into media containing a growth-limiting concentration of glucose and any one of the other carbon sources mentioned above, diauxic growth follows (see growth of wild-type in Figs. 1 and 2). As Monod (1942) showed in the case of sugar utilization by other bacterial species, the 'diauxie' observed may be interpreted on the basis that only glucose is utilized until the supply of it is exhausted, at which time the organisms then utilize the second carbon source present (see Fig. 2).

The extent of diauxic lag (the period of time intervening between the cessation of the first growth phase due to glucose exhaustion and the onset of the second growth phase due to use of the second carbon source) provides a measure of the relative magnitude of the rate of adaptation towards the second carbon source. Table 3 shows the considerable differences in the duration of this diauxic lag period with different secondary carbon sources. Glucose-grown cells adapt relatively slowly to use succinate, acetate or fumarate; furthermore, acetate delays the first log phase of diauxie. This confirms the inhibitory action of acetate upon glucose-utilization, as reported by Baskett & Hinshelwood (1949).

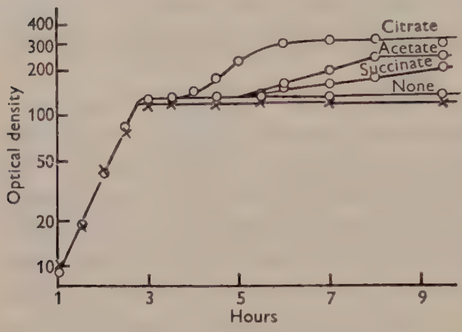

Fig. 1

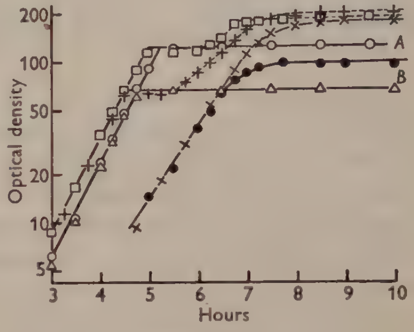

Fig. 2

Fig. 1. Growth of C+ (-○-) and C− (-×-) in aerated cultures containing 0·05 % glucose + one-half the standard concentration of one of the following: citrate, succinate, acetate, none. The C− curve shown is the same for all four types of cultures.

Fig. 2. Growth of C+ and C− in aerated glucose (G), citrate (C), and glucose + citrate cultures. C+ in 0·05 % G (-○-), in 0·05 % C (-●-), in 0·1 % C (-×-), in 0·025 % G (-△-), in 0·05 % G + 0·05 % C (-□-), and in 0·025 % G + 0·1 % C (-+-). Curve *A* is that obtained with C− in 0·05 % G and in 0·05 % G + 0·05 % C; curve *B* represents C− in 0·025 % G and in 0·025 % G + 0·1 % C. (C− data were not plotted to prevent obscuring the results.) There is no growth by C− in 0·05 % C or in 0·1 % C.

Table 3. *Growth of* C+ A. aerogenes *in aerated cultures containing glucose + a second carbon source*

Aerated media containing 0·05 % (growth-limiting) glucose + one-half the standard concentration of the indicated second carbon source were inoculated with *c.* 10^7 glucose-grown C+ (wild-type) cells. Growth was followed turbidimetrically.

Second carbon source	First growth level (utilization of glucose) (Klett units)	Second growth level (utilization of second compound) (Klett units)	Delay before second growth phase began
None	114	—	—
Glycerol	112	242	12 min.
Lactate	115	230	20 min.
Citrate	105	300	54 min.
α-Ketoglutarate	122	208	58 min.
Succinate	122	312	1½–2 hr.
Fumarate	126	240*	9 hr.
Acetate†	120	260	2–3 hr.

* Fumarate growth was continuing slowly at termination of experiment.
† Acetate caused delay in glucose-utilization of 3 hr.

Penicillin technique for screening C – *mutants*

The C – mutant of *A. aerogenes* was detected by a modification of the penicillin-screening technique originally devised for the detection and isolation of auxotrophic mutants (Davis, 1948; Lederberg & Zinder, 1948). This technique takes advantage of the fact that those cells in a culture which are actively metabolizing are more sensitive to penicillin than those which are not (Hobby, Meyer & Chaffee, 1942; Chain & Duthie, 1945). The experimental aim was to concentrate C – mutants which might be present at a low proportion in a population, by placing that population in a medium in which penicillin would selectively kill the wild-type (C +) cells, namely, in a medium containing citrate as sole carbon source.

The experimental procedure was as follows. Small samples from a glucose culture of *A. aerogenes* were inoculated into test-tubes containing the selective medium, which consisted of citrate, the usual inorganic salts, and 150 to 200 units crystalline penicillin G sodium/ml. The tubes were incubated with aeration (roller device), and samples were removed between 12 and 24 hr. A sample of the source culture of the inocula and the samples from the selective medium, were diluted and plated in glucose agar and in citrate agar. Colonies were counted after 48 and 72 hr. incubation. The purpose of this plating was twofold: (1) to be able to isolate the separated clones of the cells surviving the penicillin screening; (2) to determine whether the screening procedure was efficient. The procedure could be presumed efficient if a significant discrepancy in the colony count existed between the glucose and citrate agar plates made from the penicillin-screened population and when none existed between those made from the source culture.

Since a ratio of the glucose agar count to the citrate agar count (the G:C ratio) greater than 1·5 is significant, the finding of such or higher ratios reveals the existence of heterogeneity in the population with respect to the ability to grow in citrate agar. It then remains to be discovered to what the heterogeneity can be attributed. To determine whether the heterogeneity could be accounted for on the basis of the proportion of stable C – mutants present in the population, colonies were picked from the glucose agar plates and tested for their ability to grow in defined media containing citrate as sole carbon source. This test was made in two ways: (1) by streaking directly on the surface of glucose and of citrate agar; (2) by inoculating *c.* 10^6 to 10^7 cells into liquid glucose and liquid citrate media. Those isolates characterized by ability to use glucose and inability to grow in citrate media are referred to as 'C – isolates'. If the heterogeneity observed in a given experiment were due solely to the proportion of C – mutants, then the G:C ratio should be equivalent to the ratio of total number of glucose isolates tested to the difference between this number and the number of C – isolates obtained.

An investigation of various populations, differently grown or treated, provided the following results (see also Table 1). Significant G:C ratios were never observed in platings of: (1) glucose cultures in the log phase; (2) stationary-phase glucose cultures which had stopped growing due to exhaustion of glucose. Significant differences were consistently found in platings of: (1) unaerated, acid-limiting glucose cultures in the stationary phase; (2) log-phase cultures subjected to ultraviolet irradiation; (3) populations surviving penicillin treatment. Stable C− mutants were found, however, only in penicillin-screened populations. Despite the considerable heterogeneity in irradiated populations and in unaerated stationary-phase, acid-limiting glucose cultures, no C− mutants were ever recovered by the method just described of testing the glucose agar isolates. Indeed, even in the case of penicillin-screened populations, it was common to find that the G:C ratio was much greater than could be explained by the proportion of C− mutants recovered. Thus, in addition to genetic mutants, some apparently readily reversible modification, manifested by inability to grow in citrate agar, occurs in glucose-grown populations of *A. aerogenes*. This is extremely important technically in the penicillin-screening procedure, for it is necessary to utilize homogeneous glucose cultures as the source of populations subjected to screening. Since in populations exhibiting non-genetic heterogeneity the vast majority of the cells incapable of growing on citrate are not mutants, penicillin screening of such populations leaves the proportion of mutants so low as to render the procedure impracticable for the recovery of these mutants. For this reason, aerated log-phase glucose cultures were always used in our experiments as the source cultures for the screening of C− mutants.

In four different penicillin-screening experiments thirty-two independently isolated C− mutants were obtained. The results of two of these experiments are given in Tables 4 and 5. Table 4 presents the results of an experiment in which the source culture was also subjected to a short dose of ultraviolet irradiation. The results indicate that there was homogeneity for ability to grow in citrate agar in the unirradiated population and in the irradiated population that underwent three or four divisions in aerated glucose medium. There was, however, a heterogeneity in the irradiated population that cannot be accounted for on the basis of the presence of C− mutants. There was heterogeneity in the populations which survived 16 hr. in the selective media that can be explained by the proportion of the C− mutants in those populations. This experiment was the most successful experiment performed for the recovery of C− mutants.

More typical results are those summarized in Table 5 from an experiment which was essentially the same as that summarized in Table 4 except that (1) no irradiation was carried out, (2) the size of the inoculum into the sterilizing medium was smaller, (3) the population surviving in the selective medium was plated before and after washing with saline. This experiment revealed the typical finding with populations which survive penicillin treatment, namely, that most of the heterogeneity is due to cells whose inability to grow on citrate is not an inherited and stable character. Nevertheless, some C− isolates were obtained even where non-genetic heterogeneity existed in penicillin-treated populations. It is also clear that washing the surviving populations had no effect on the total viable count or on the G:C ratio.

Table 4. *Detection and isolation of* C− *mutants of* A. aerogenes (*Exp.* 1)

The source culture was an aerated glucose (0·05 %) culture in the log phase. Viable counts of a diluted sample of the source culture were determined on glucose agar and on citrate agar at time of removal of inocula for various treatments. Viable counts of diluted samples of variously treated populations were similarly obtained by plating on glucose and citrate agar. The selective medium consisted of the salts of the defined medium + the standard concentration of citrate + 200 units crystalline penicillin G sodium/ml.

	Counts on				
Population tested	Glucose agar (G) (Bacteria/ml. sample)	Citrate agar (C) (Bacteria/ml. sample)	Ratio G : C	No. of glucose colonies tested	No. of C − mutants
Unirradiated source culture (U)	$1\cdot42\times10^{8}$	$1\cdot30\times10^{8}$	1·1	20	0
Irradiated source culture (I)	$1\cdot81\times10^{7}$	$0\cdot60\times10^{7}$	3·0	20	0
Irradiated culture after aeration in glucose medium (IG)	$1\cdot82\times10^{8}$	$1\cdot61\times10^{8}$	1·1	20	0
U population in selective medium at:					
Zero time (0 hr.)	$23\cdot3\times10^{5}$	$21\cdot3\times10^{5}$	1·1	—	—
16 hr.	$12\cdot8\times10^{3}$	$4\cdot0\times10^{3}$	3·2	14	13
IG population in selective medium at:					
Zero time (0 hr.)	$29\cdot8\times10^{5}$	$26\cdot4\times10^{5}$	1·1	—	—
16 hr.	$20\cdot5\times10^{3}$	$2\cdot8\times10^{3}$	7·3	14	12

Table 5. *Detection and isolation of* C− *mutants of* A. aerogenes (*Exp.* 2)

The source of inoculum was an aerated glucose (0·05 %) culture in the log phase. Viable counts of a diluted sample of the source culture were determined on glucose agar and on citrate agar at time of removal of inoculum for selective medium. Viable counts of diluted samples of selective medium were similarly determined immediately after inoculation and at 19 hr. after inoculation. The expected C − proportion was calculated as the proportion of cells forming colonies on glucose agar which did not form colonies on citrate agar.

	Counts on		
	Glucose agar (G) (cells/ml.)	Citrate agar (C) (cells/ml.)	Ratio G : C
Viable count, inoculum source	$8\cdot6\times10^{7}$	$8\cdot2\times10^{7}$	1·0
Viable count from selective medium at:			
0 hr.	$3\cdot7\times10^{5}$	$3\cdot5\times10^{5}$	1·0
19 hr. (washed)	$13\cdot6\times10^{2}$	$2\cdot65\times10^{2}$	5·1
19 hr. (unwashed)	$12\cdot3\times10^{2}$	$2\cdot45\times10^{2}$	5·0

			C − proportion	
	No. of colonies tested	No. of C − mutants	Observed	Expected
C − proportion of surviving population	32	2	0·06	0·81

Stability of wild-type and mutant strains

All the physiological experiments described below were performed with two of the C− isolates obtained from screening the unirradiated population indicated in Table 4. These two C− isolates, as well as the wild-type strain of *A. aerogenes*, were maintained for 200–300 generations in aerated nutrient broth and in aerated glucose cultures (25 ml. of medium contained in 125 ml. Erlenmeyer flasks). Serial transfer was effected by diluting a sample of a previous culture which had stopped growing, so that *c.* 50 cells were transferred into a flask of sterile medium. Approximately twenty-six generations of growth took place in each flask culture. At the beginning and at the end of the serial transfer, *c.* 10^6 cells were tested for growth in defined medium containing citrate as sole carbon source. The C+ (wild-type) strain retained its ability to utilize citrate as the sole carbon source and the C− strain remained unable to utilize citrate. This result was obtained whether aerated nutrient broth or aerated glucose was employed as the medium for serial transfer. One may conclude, therefore, that the citrate-utilizing character in the C+ (wild-type) strain is a stable inherited character, as is the inability of the C− mutant to utilize citrate. We are justified, therefore, in considering the C− strain as a stable genetic mutant of *A. aerogenes*.

Non-genetic loss of ability to grow in media containing certain carbon sources

Populations of *A. aerogenes* which have stopped growing in unaerated 0·5% glucose cultures due to the development of a low pH value become heterogeneous with respect to ability to grow in citrate agar. The percentage of cells unable to grow in citrate agar is a function of the time they are exposed to the growth-limiting conditions in the stationary phase (Fig. 3). The rate of increase of these cells is exponential; the percentage of cells which cannot grow in citrate agar doubles approximately every 5 hr. An interpretation of the kinetics of this phenomenon is presented elsewhere (Ravin & Norman, 1952).

In a certain experiment a heterogeneous culture, after 20 hr. in the stationary phase, contained *c.* 12 times more cells that would grow in glucose than would grow in citrate agar. Yet when forty-eight of the glucose colonies were tested for citrate-utilization (as described in the section on mutant screening), each one grew as well in defined citrate media as did the control colonies isolated from the citrate agar plates. Identical results were obtained in several similar experiments. Clearly the loss of ability to grow in citrate medium which is induced in unaerated stationary-phase 0·5% glucose cultures is extremely unstable.

The following experiment was performed to rule out back-mutation and selection of C+ progeny as the basis of this instability. A heterogeneous glucose culture was washed and samples were removed, before and after washing, for plating and for inoculation into tubes of defined medium. These tubes, which either lacked a carbon source or contained 0·5% glucose, were incubated without aeration. Samples from the tubes were plated after 1½ and 4 hr. Some of the platings were made on succinate and lactate agars as well as on the usual glucose and citrate agars. The results are given in Table 6. Homogeneity was fully restored in 4 hr. in the unwashed population as well as in the washed population provided with a carbon source.

Table 6. *The restoration of homogeneity in a heterogeneous glucose culture of* A. aerogenes

Sample populations from an unaerated stationary-phase glucose (0·05 %) culture were inoculated into defined media under the conditions shown, and the restoration of homogeneity to the populations was followed by platings on glucose, citrate, succinate and lactate agars.

			Colony counts in			
		Time	Glucose agar	Citrate agar	Succinate agar	Lactate agar
Inoculum	Medium	(hr.)	(Bacteria × 10^5/ml. sample)			
Unwashed population from source culture	Salts minus glucose	0	74·3	10·6	0·17	67·7
		1·5	151·0	107·0	—	—
		4	225·0	190·0	156·0	229·0
Washed population from source culture	Salts minus glucose	0	51·1	9·7	0·13	54·4
		1·5	84·0	52·0	—	—
		4	75·0	39·0	9·0	71·0
Washed population from source culture	Salts plus glucose	0	51·1	9·7	0·13	54·4
		1·5	88·0	60·0	—	—
		4	160·0	180·0	173·0	190·0

Since the mean generation time of *A. aerogenes* in unaerated glucose media is 43 min., at most only two generations can take place in 1½ hr. and about five generations in 4 hr. If the cells unable to grow in citrate or in succinate media were stable mutants, with a reversion rate, say, of 10^{-4} to 10^{-6}/generation/cell, then a selection hypothesis would have to explain the increases in the numbers of C+ and S+ cells as being due to the growth of these cells. However, such growth increases as were actually observed, namely, a tenfold increase in 1½ hr. of the number of cells able to grow in citrate and a 900-fold increase within the same time of the number of cells able to grow in succinate, are patently impossible. One can only conceive of the restoration of homogeneity as a process whereby those cells initially incapable of growing on citrate (or succinate) regain this ability.

In the experiment summarized in Table 6 it may be seen that cultures heterogeneous for ability to grow in citrate are also heterogeneous for ability to grow in succinate, but homogeneous for ability to grow in lactate. Subsequent experiments, involving the plating of heterogeneous glucose cultures in media containing different carbon sources, revealed that the non-genetic modification affects ability to grow in media containing as sole carbon sources those substrates to which C+ (wild-type) slowly adapts. The ability to grow in glycerol, lactate, pyruvate or malate media is unaffected, whereas the ability to grow in citrate, succinate, α-ketoglutarate, acetate or fumarate media is lost.

Comparison of growth of C+ *and of* C− *strains in nutrient broth*

In studies of the comparative physiology of the wild-type and mutant strains of *A. aerogenes*, all experiments were performed at least twice; in the original experiment one C− isolate was used, and in the repeat the second isolate was generally used. Identical results were always obtained.

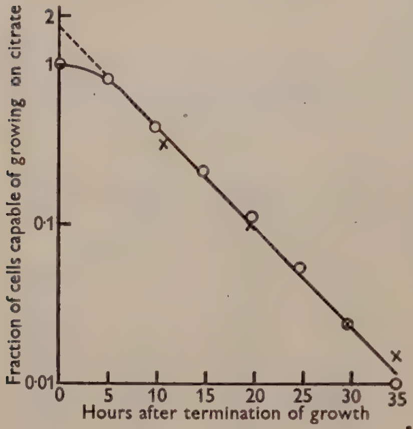

Fig. 3

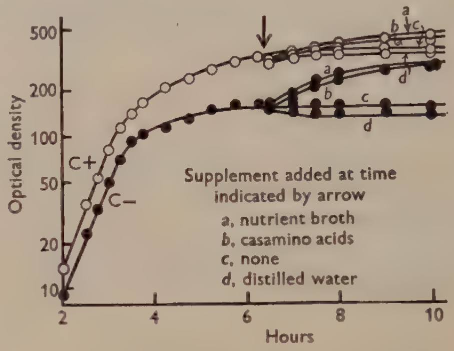

Fig. 4

Fig. 3. Fraction of population (in growth-terminated unaerated glucose culture) capable of growing in citrate agar; as a function of time after termination of growth. Values plotted are calculated from colony-count data as the fraction of cells capable of growing in glucose agar which are also capable of growing in citrate agar. × = data from a single experiment; ○ = averaged results from five different experiments. The ordinate scale is logarithmic.

Fig. 4. Growth of C+ (-○-) and C− (-●-) in aerated nutrient broth cultures. Cultures received supplement at time indicated by arrow.

Fig. 4 shows the results of an experiment in which the growth of C+ and of C− was followed in aerated nutrient broth cultures. Approximately 10^7 cells from glucose-grown populations were inoculated in the usual way into each of four tubes containing 6 ml. nutrient broth. After growth had stopped, 1·0 ml. of sterile 6 × concentrated nutrient broth was added to one tube, 1·0 ml. of sterile 12 % (w/v) Casamino acids (Difco) was added to another, and 1·0 ml. of sterile distilled water was added to a third tube which was used as a control for the effect of dilution. The fourth tube received no addition of any kind.

The following facts can be noted:

(1) C+ and C− grow at the same rate during the log phase (generation time 28–30 min.); (2) C+ attains a higher level of growth (*c*. 350 Klett units) than does C− (*c*. 150 Klett units); (3) the growth of both C+ and C− can be increased by the addition of substances present in nutrient broth, and is therefore apparently limited by the exhaustion of these substances. These substances are replaceable by the amino-acids in the acid digest of casein (Casamino acids). The hypothesis is proposed that there are substances in

nutrient broth which can be utilized by C+ but not by C−. The amount of citric acid in nutrient broth could hardly account for the difference in the amounts of growth of C+ and C−. Therefore, if this hypothesis is correct, C− must also be incapable of utilizing certain compounds, other than citrate, which are present in nutrient broth.

Metabolites utilized by C+ *and* C− *strains*

Loss of ability of C− *strain to utilize substrates to which* C+ *slowly adapts.* To test the hypothesis suggested by the comparison of the growth of C+ and C− in nutrient broth, we examined the ability of C− to utilize the carbon sources previously investigated with wild-type C+. The tests were made in two ways: (1) by streaking *c.* 10^8 to 10^9 cells from nutrient agar slopes on to the surface of defined media solidified by washed agar; (2) by inoculating *c.* 10^6 to 10^7 glucose-grown cells into aerated tubes of liquid-defined media. Any given test medium contained only one carbon source (except the agar in solid media), namely, the compound the utilization of which was being investigated.

In the liquid cultures, growth of the C+ (wild-type) strain, used as control, was like that described in the section on 'Adaptation' above. But the C− mutant did not grow at all in cultures containing citrate, α-ketoglutarate, succinate, fumarate, acetate, glutamate or aspartate as sole carbon source. In liquid media containing glucose, glycerol, malate, pyruvate, lactate or alanine, C− grew with the same very brief lag period shown by the C+ wild-type.

The results obtained by the agar streak technique corroborated those obtained with the aerated tube cultures. With the surface streaks, however, lag was indicated by the length of time before appreciable surface growth was visible over the light background smear of cells initially spread on the surface. Even where the lag period was long, as with fumarate agar, growth of C+ always appeared homogeneously over the surface of the agar. The entire background smear developed continuously into the final surface growth, and papillations never occurred.

The appearance of the C− mutant streak on fumarate agar was different. Within the streaked area, several (8 to 10) papillae appeared at about 5 days of incubation; these papillae grew gradually into large surface colonies which could be isolated and tested. It was presumed that the papillae were clones developing from mutants present in the original heavy streak inoculum, which mutants had regained the ability to utilize fumarate. Occasionally in these experiments one or two such papillae appeared on the surfaces of citrate, α-ketoglutarate or succinate agars which had been heavily streaked with C− cells (see below).

A summary of the results of these substrate-utilization tests is given in Table 7, which shows that a wide variety of substrates utilizable as carbon sources for the growth of the wild-type (C+) were not utilized by the C− mutant. Furthermore, the class of compounds not utilized by C− corresponds to the class of compounds not utilized by glucose-grown C+ cells until after an appreciable lag period. The smaller amount of growth by C− in nutrient broth is now explainable on the ground that two carbon sources present in nutrient broth in large quantities, aspartate and glutamate, cannot be utilized by C− for growth. It is also conceivable that other amino-acids present in nutrient broth cannot be utilized by C− because, in order to be utilized, they must be metabolized via a pathway involving some of the compounds not utilizable by C−.

Table 7. *Substrates utilized by* C+ *and by* C− *strains of* A. aerogenes

Utilization of substrate was determined by presence or absence of growth in liquid cultures in defined medium containing indicated substrate as sole carbon source and by streak cultures on surfaces of similar media solidified with washed agar. Of the compounds listed the ones in the right-hand column are those to which C+ (wild-type) cells slowly adapt after being previously grown on glucose.

Substrates utilized by C+ and by C− strains	Substrates utilized only by C+ strain
Glucose	Fumarate
Glycerol	Aspartate
Pyruvate	Succinate
Lactate	Acetate
Alanine	Glutamate
Malate	α-Ketoglutarate
	Citrate

Behaviour of C− *mutant with respect to fumarate and malate.* To verify the behaviour of the C− mutant with respect to malate and fumarate, two other independent methods were employed. First of all the S− strain of *Esch. coli* K12 (Nelson, 1949) was used as an assay organism. Fumarate and malate agar were prepared in the usual way and different sectors of the agar were streaked with *A. aerogenes* C+ (wild-type), with *A. aerogenes* C− mutant, with *Esch. coli* S+ or with *Esch. coli* S−. As Nelson has previously reported, *Esch. coli* S+ is F+M+ (fumarate- and malate-utilizing), whereas the S− strain is F−M−. On the same test agars, *A. aerogenes* C+ was F+M+, whereas C− was F−M+. Two sets of media were prepared containing the standard concentration of malate as sole carbon source; one set was sterilized by autoclaving and the other by filtration through a sintered glass filter. The growth of C+ and of C− in aerated tubes of the two media was followed turbidimetrically. No differences were observed in any of the growth characteristics (rates, final amounts, etc.) of C+ or of C− in either type of medium. There seems little reason to doubt, therefore, that the C− mutant is truly capable of utilizing malate while incapable of utilizing fumarate.

Comparison of growth of C+ *and* C− *strains in glucose media*

Growth in aerated glucose cultures. A comparison was made between the growth of the C+ and C− strains in media containing glucose as sole carbon source. Fig. 5 gives the results of one of numerous similar experiments in which glucose-grown inocula were aerated in tubes containing glucose (0·5 or 0·05%) medium. It is clear that in cultures where exhaustion of glucose limited growth no difference could be detected between either the rates or the final amounts of growth of C+ or C−. In cultures where acid pH values caused growth to stop, the rates of growth of C+ and C− were the same, although C− consistently showed a somewhat higher final amount of growth than C+. Sometimes this difference was significant, but not always. The average values are given in Table 8. It may be definitely stated that C+ utilizes glucose for growth no more efficiently than does C−, although C+ is capable of utilizing as sole carbon sources several important energy-yielding metabolites which C− cannot utilize.

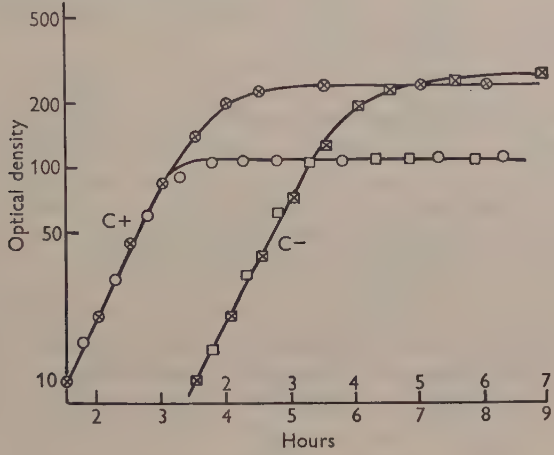

Fig. 5. Growth of C+ and C− in aerated glucose cultures. C+ in 0·05 % (-○-) and 0·5 % (-⊗-) glucose; C− in 0·05 % (-□-) and 0·5 % (-⊠-) glucose. The C− growth curves are displaced 2 hr. to the right to permit comparison with C+ curves.

Growth in cultures containing glucose and another carbon source. Glucose-grown populations of C+ and of C− were inoculated in the usual way into media containing 0·05 % glucose + one-half the standard concentration of citrate, succinate or acetate. The cultures were aerated and the growth was followed turbidimetrically. The results are shown in Fig. 1. The second growth period, corresponding to that in which citrate, succinate or acetate was being utilized by C+, is completely lacking for C−. This means that the C− mutant cannot utilize citrate, succinate or acetate even in cultures which contain a second utilizable carbon source.

Proof that the first growth period is the one in which glucose is utilized and that the second growth period is the one in which the second carbon source is utilized, is given by the results of the experiment illustrated in Fig. 2. In this experiment C+ and C− were grown in media containing varying proportions of glucose and citrate (0·05 % glucose + 0·05 % citrate or 0·025 % glucose + 0·1 % citrate). Comparison of C+ controls, containing only glucose (0·05 or 0·025 %), with C+ cultures containing both carbon sources, provides the conclusive evidence. It should be noted, furthermore, that the C− cultures containing the citrate supplement are no different from the C+ controls containing only glucose.

Effect of aeration upon utilization of carbon sources

The possibility existed that certain of the compounds (other than glucose), which were utilizable by C+ and by C−, could not be as efficiently utilized by C− as by the C+ strain. To test this the following experiment was performed. The usual inocula from glucose cultures of C+ and of C− were made into quadruplicate sets of media containing, respectively as sole carbon source, standard concentrations of glucose, pyruvate, malate, citrate or succinate. Two tubes of each medium were incubated with aeration and two without aeration. The final amounts and rates of growth were measured. Typical results are recorded in Table 8.

Table 8. *Effect of aeration upon the efficiency of utilization of various substrates by* C+ *and by* C− *strains. of* A aerogenes

Tube cultures identically inoculated and containing the indicated compound as sole carbon source were either unaerated (standing cultures) or aerated on a roller device. Incubation was at 37°. Growth was measured turbidimetrically. Rates of growth in pyruvate media were not calculated because of insufficient growth in the visible range of turbidity.

	Strain C+		Strain C−	
Carbon source	Rate of growth (mean generation time; min.)	Final amount of growth (Klett units)	Rate of growth (mean generation time; min.)	Final amount of growth (Klett units)
Glucose:				
Aerated	32	310	32	345
Unaerated	40	155	41	165
Pyruvate:				
Aerated	—	67	—	33
Unaerated	—	32	—	33
Malate:				
Aerated	34	210	36	145
Unaerated	122	76	119	74
Citrate:				
Aerated	48	240	0	0
Unaerated	135	86	0	0
Succinate:				
Aerated	*c.* 4 hr.*	350†	0	0
Unaerated	*c.* 25 hr.*	350†	0	0

* The lag period prior to logarithmic rate of growth was *c.* 24 hr. in unaerated as compared to *c.* 48 hr. in aerated succinate medium.

† Final levels not accurately determined because of brown discoloration of succinate medium at growth levels greater than 350 Klett units.

The striking effect of aeration with any substrate was to increase the rate of growth. As with cultures growing on succinate, the increase in rate of growth by aeration could be observed even when the lag period was appreciably shorter in unaerated media. It was also clear that the C− strain could not utilize citrate or succinate whether or not the cultures were aerated. This finding was verified for each of the substrates that C− cannot utilize.

Aeration raised the final level of growth of the C+ (wild-type) strain above that in unaerated media. This effect did not occur when C− was utilizing pyruvate; in this case the final amounts of growth were the same in aerated and in unaerated cultures. One other finding needs emphasis: the final amounts of growth that C+ attained in aerated pyruvate and malate cultures were greater than those reached by C−. In unaerated pyruvate and malate cultures, however, C+ and C− reached the same final amount of growth. These experiments with pyruvate and malate were performed three times with results which were reproduced within the 5 % experimental error observed in the measurements of any single experiment. It may be concluded, therefore, that aeration permits C+ to utilize pyruvate and malate more efficiently than C−. This greater efficiency is reflected neither in the rate of growth nor in the length of the lag phase, but in the final amount of growth. This fact renders unlikely any permeability differences between C+ and C−, since such differences would be expected to influence the lag period or rate of growth.

Reversions of the C− *mutant*

Endo reaction. All thirty-two independently isolated C− strains were alike with respect to the range of substrates which they utilized. In addition, all these strains were methyl-red negative, incapable of producing indole in tryptone broth (Difco), incapable of growing or producing indole in defined medium containing L-tryptophan as sole carbon source, and resistant to all the coli bacteriophages of the T series. In these respects, the C− strains of *A. aerogenes* are exactly like the C+ (wild-type) and unlike wild-type strains of *Esch. coli.* An interesting difference exists, however, in the growth characteristics of the C+ and C− strains on Endo agar. When a light surface streak is made on Endo agar, no difference in the appearance of the growth of C+ or C− can be noted during the first 12 hr. Both show a pinkish surface growth and only slight staining of the medium. This appearance is in sharp distinction to the dark red growth of *Esch. coli* on Endo agar, associated with an intense reddish purple staining of the medium. After 24 hr. (when *Esch. coli* growth and stain become extremely marked), the C+ growth continues to increase, and although the growth becomes magenta red in colour, there is little change in the colour of the medium. The C− growth, on the other hand, ceases within 24 hr. and the medium gradually acquires a dark red colour which, however, never gets as intense as that due to *Esch. coli.* Thus, not only can a sharp distinction be made between the type of growth of *Esch. coli* and *A. aerogenes* on Endo agar, but a nice distinction can also be made between the 24 and 48 hr. surface streaks of C+ and C− on this medium.

The difference in amounts of growth of C+ and C− on Endo agar is probably explained by the fact that C+ gives a greater final amount of growth on nutrient broth than does C−; Endo agar contains a nutrient broth base. The greater accumulation in the C− cultures of an aldehydic substance which combines with the Schiff reagent (bisulphite-basic fuchsin) in Endo agar is noteworthy because it may throw some light on the nature of the metabolic block in the C− strains.

Reversion pattern of C−. Papillae isolated from the surface streaks of the C− strains on agar in the substrate-utilization tests were incubated on nutrient agar slopes and re-tested by streaking some of the growth on a number of single carbon source agars. Papillae recovered from C− streaks on citrate, α-ketoglutarate and succinate agars fell into two classes: (1) those consisting of cells exactly like C+ (wild-type) in the type of Endo reaction and in the range of substrates utilized; (2) those consisting of 'slow growers'. The latter were characterized by a long delay, slower growth, and occasionally not as heavy a growth as that of C+ (wild-type) on the test agars. There was a good correlation between the slow type of growth and the time of appearance of the papilla, the isolate of which showed that type of growth when tested. When a papilla appeared after the third or fourth day of incubation, it was always a 'slow grower' on test. There was also a correlation between 'slow growth' and the type of Endo reaction, for all 'slow growers' gave an Endo reaction like that of C−. 'Slow growers' utilized all the substrates that C+ (wild-type) could utilize, and were distinguishable from the latter only by the obvious difference in rate of growth and in type of Endo reaction.

Table 9. *Testing of papilla-isolates by surface agar streak method*

Slope cultures derived from papillae isolated in agar streak tests of mutant strains were re-tested by streaking across the surfaces of defined media solidified with washed agar and containing, respectively, the following compounds as sole carbon sources: glucose (G), citrate (C), α-ketoglutarate (α-KG), glutamate (Glut), succinate (S), fumarate (F), aspartate (Asp). In Test I aspartate and glutamate were omitted. The C+ (wild-type) control utilized all these compounds; the C− mutant control utilized only glucose.

Mutant strain tested	Papillae isolated from defined agar containing	No. of papillae tested	Type of Endo reaction	Substrates utilized by papilla-isolates
Test I: C−	F	15	C−	G, F
	F	1	C−	G; all others slow
	C	13	C−	G; all others slow
	C	3	C+	All
	α-KG	12	C−	G; all others slow
	α-KG	1	C+	All
	S	14	C−	G; all others slow
	S	1	C+	All
Test II: C−	F	8	C−	G, F, Asp
Test III: C− F+	C	3	C+	All
	C	1	C−	G; all others slow
	α-KG	8	C+	All
	S	1	C+	All
	S	2	C−	G; all others slow
	Glut	1	C+	All
	Glut	2	C−	G; all others slow

Papillae recovered from C− streaks on fumarate agar also fell into two classes, one of which, however, consisted of cells which had regained ability to utilize fumarate and aspartate but none of the other compounds not utilized by C –; the other class consisted of 'slow growers'. Papillae were never observed on acetate agar streaks. The reverted strains which were incapable of utilizing citrate but capable of utilizing fumarate (C – F +) possessed, like the 'slow growers' (C*), a C – type of Endo reaction (Table 9).

The C – F + reverted strains recovered from fumarate agar were then streaked in the usual way on citrate, α-ketoglutarate, succinate and glutamate agars; papillae which appeared were isolated and tested. Two classes of isolates were again obtained: (1) prototrophic; (2) 'slow growers'. A summary of the papilla-testing procedure and some typical results are recorded in Table 9. From these results, it is possible to construct a scheme of the pattern of mutation and reversion in *A. aerogenes* strain 211, which is given in Fig. 6.

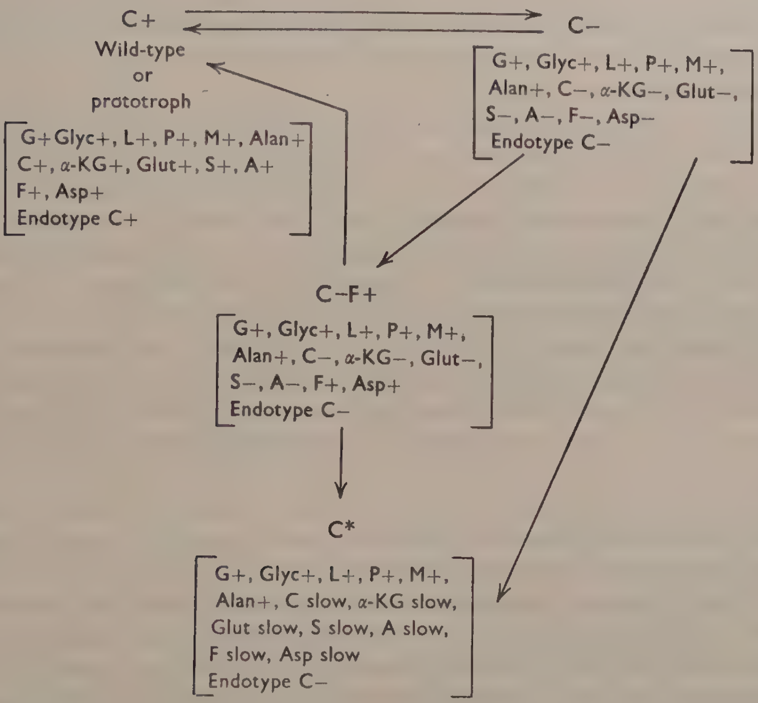
C+
Wild-type
or
prototroph
G+Glyc+, L+, P+, M+, Alan+
C+, α-KG+, Glut+, S+, A+
F+, Asp+
Endotype C+
C–
G+, Glyc+, L+, P+, M+,
Alan+, C–, α-KG–, Glut–,
S–, A–, F–, Asp–
Endotype C–
C–F+
G+, Glyc+, L+, P+, M+,
Alan+, C–, α-KG–, Glut–,
S–, A–, F+, Asp+
Endotype C–
C*
G+, Glyc+, L+, P+, M+,
Alan+, C slow, α-KG slow,
Glut slow, S slow, A slow,
F slow, Asp slow
Endotype C–

Fig. 6. **Pattern of mutation and reversion in *Aerobacter aerogenes* with respect to the utilization of the Krebs cycle compounds. 'Endotype' refers to type of reaction shown by growth on Endo agar.**

The various prototrophic and mutant types have been subjected to single colony isolation and maintained on nutrient agar slopes by serial transfer, and they have not lost their distinguishing characteristics. No physiological studies of the reverted strains comparable to those performed with the C – mutant have yet been made. Consequently, the C* or slow-growing class has not been studied sufficiently to provide an understanding of the nature of the 'slow growth'.

DISCUSSION

A certain class of carbon sources, which includes citrate, succinate, acetate, fumarate and α-ketoglutarate, cannot be readily utilized by glucose-grown populations of *A. aerogenes*. This is reflected in a comparatively long lag period before the attainment of a logarithmic rate of growth by glucose-grown populations when inoculated into media containing one of these other substrates as the sole carbon source. The nature of this adaptive process is not yet understood, but the ensemble of the following facts makes it unlikely that we are dealing in this case with the selection of rare mutants in the glucose-grown populations:

(1) Every cell present in a log-phase glucose-grown population produces a colony when plated on a medium of washed agar + one of the aforementioned compounds as carbon source. Since log-phase glucose-grown C − mutant cells do not form colonies when plated on the same medium, no contaminating material in the agar appears to be available for the growth of cells incapable of utilizing the particular carbon source added to the medium.

(2) Eight generations of growth in glucose medium are enough to require that a population derived from cells that were originally adapted to one of the aforementioned compounds should re-adapt to this compound. This indicates that adapted cells are not mutants which inherit ability to utilize the compound in question, but rather are transitorily, physiologically altered, cells.

The present case, therefore, seems to be one of physiological adaptation by the *individual* cells exposed to succinate, or acetate, etc., after being previously grown on glucose. The results obtained in this investigation have shown that this adaptive process is susceptible to two kinds of obstruction. One is non-genetic; that is, it is not inherited by the cells in which the obstruction occurs. When one of these cells is inoculated into a medium in which it can divide, the obstruction disappears and the cell is once more capable of adaptive growth. The other obstruction is stable, for a cell in which it occurs gives rise to progeny that do not regain the lost adaptive ability.

Adaptation involves the setting into action of a complex of biochemical reactions, and these, according to Monod (1950), must at some point require the intervention of the genetic apparatus of the cell. In wild-type cells the usual effect of mutation is to prevent, in some way, this complex of reactions from occurring. The nature of the reactions blocked in the C − mutant of *A. aerogenes* cannot be determined from the results so far obtained. It seems likely, however, that at least one of these reactions is an aeration-dependent reaction in the dissimilation of pyruvate, since the growth of C + and of C − on pyruvate is identical except when aerated. The results also justify the assumption that the reactions blocked in the mutant do *not* occur in wild-type *A. aerogenes* when growing on glucose, for such an assumption will account at once for two of the present findings; namely, that the growth of C − in glucose cultures is the same as that of C + ; and that the class of compounds which C − cannot utilize coincides exactly with the class of compounds to which the C +

strain slowly adapts after growth on glucose. The reactions that are slowly set in action in wild-type cells which are inoculated into media containing citrate or succinate, etc., after having previously grown on glucose, are probably the ones that are completely blocked in the C− mutant. The question arises whether these reactions, genetically blocked in the C− mutant and not occurring in wild-type cells during their growth on glucose, include reactions in the Krebs cycle. One may conclude that either the Krebs cycle does not operate during glucose dissimilation or that the utilization of exogenous citrate, succinate, acetate, etc., by cells previously grown on glucose requires at the outset certain reactions that bring these substrates into the Krebs cycle. It is conceivable, for example, that these substrates must be coupled to a specific compound in order to be metabolized via the Krebs cycle, and that the coupling reactions are the ones blocked in the mutant and involved in adaptation.

That the substrates involved in the non-genetic effect correspond to the substrates not utilizable by the C− mutant suggests some close relationship between the non-genetic effect and the mutation. In this connexion, it is significant that the kinetics of the induction of the non-genetically modified cells can be interpreted on the basis of the inactivation of 1 or 2 particles/cell (Ravin & Norman, 1952). Finally, the fact that ultraviolet radiation can induce the non-genetic effect (Ravin & Norman, 1952) suggests that material containing nucleic acid may be the site of this action.

The mutation from C+ to C− in *A. aerogenes* is of taxonomic interest because it affects a character that is used to distinguish this species from *Esch. coli.* Whatever may be the nature of the block engendered by this mutation, it is undoubtedly not the same as that which prevents the utilization of citrate in *Esch. coli,* because a comparison of the substrates utilized by C+ and C− strains in both species (*A. aerogenes* 211 and *Esch. coli* T16) reveals that, whereas in *Esch. coli* only the utilization of citrate is affected by mutation, in *A. aerogenes* the utilization of a wide variety of other 'Krebs cycle compounds' is also affected. Nevertheless, the step-wise regaining of ability to utilize some of these other compounds, as illustrated by the C− F+ reversion, indicates how, in the course of evolution of an originally C+ *A. aerogenes* strain, a condition could be attained approximating that of *Esch. coli* with respect to the utilization of 'Krebs cycle compounds'. In any case, the existence of mutations affecting characters that are used to distinguish between species supports the view that among bacteria, as well as among higher organisms, mutations serve as the raw materials for biochemical evolution.

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Ultraviolet Induced Mutations in *Chlamydomonas moewusii* Gerloff

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SUMMARY: Mutations were induced in the unicellular green alga *Chlamydomonas moewusii* Gerloff by ultraviolet irradiation. The majority of mutants detected were slow-growing types which did not respond to organic supplements in the medium, and palmelloid forms in which flagellated cells were not produced, or only occurred in small numbers. Among biochemical mutants, one exhibited impairment of the photosynthetic mechanism, and two were vitamin-dependent, respectively requiring *p*-aminobenzoic acid (PABA) and aneurin for growth. Some mutants were distinguished by features of cell contents, cell form, or mode of cell division, while in others motility of the cells was restricted by the absence or paralysis of the flagella.

Though it is of some importance to investigate physiological and genetical processes in green plants by the use of a micro-organism with a comparatively short life cycle, there have been very few genetic studies carried out with algae. The bulk of the literature in this limited field is the work of F. Moewus, and his reported results, almost without exception, have remained unconfirmed.

Mating types of *Chlamydomonas moewusii* were isolated by Dr L. Provasoli in 1948, and the physiology and life cycle of this organism have been studied (Lewin, J. C., 1950; Hutner & Provasoli, 1951; Lewin, R. A., 1952*a*). The organism is completely—and, it seems, obligately—autotrophic, grows well on mineral agar media, and in suitable conditions the cell number will double in 12 hr. The vegetative cells are haploid. Gamete formation can be readily induced, and zygote formation and germination may be controlled experimentally (Lewin, R. A., 1949). Meiosis takes place within the zygospore during germination; the haploid cells which emerge can be separated (Moewus, 1940*a*), and from these cells new vegetative clones can be established.

The species is regularly heterothallic: the mating-type character segregates in the expected 2:2 or 4:4 fashion, as in *Dunaliella salina* (Lerche, 1937) and *C. Reinhardi* (Smith & Regnery, 1950). Since the two original clones of complementary mating-type (here designated *plus* and *minus*, or WT+ and WT−) appear identical except for mating behaviour (Lewin, R. A., 1950*a*), it was necessary to obtain other marker genes in order to examine the genetics of the organism. The genetic data are presented elsewhere (Lewin, R. A., 1952*b*).

Lerche (1937) found that *D. salina oblonga*, which she had isolated from natural sources, differed from *D. salina salina* in a single gene. In *C. moewusii*, the frequency of recognized spontaneous mutation appears to be fairly low. (In one experiment, among *c.* 1200 colonies examined only eight were found which in size or contour differed in any way from the rest. Of these, two were persistently aberrant in subculture: one produced slightly drier colonies than normal, while the other—which subsequently reverted twice to the wild-type—grew slowly as a palmella on all media.) Henceforth in this paper only 'induced' mutations will be considered. Proof of the experimental induction of any given mutant cannot be presented, and the epithet 'induced' is used loosely, to cover all changes detected after irradiation—i.e. *post hoc*, not necessarily *propter hoc*.

Gerloff (1940) attempted to induce mutations in *C. terricola* and *C. humophilos* by ultraviolet light. He used a Hanau ultraviolet source (S.N. 300) at a distance of 35 cm. for durations between 20 sec. and 5 min., but was unable to detect any morphological or sexually aberrant mutants. After X-irradiation dosages of 1500, 3000 and 6000 r. of vegetative cells and zygotes, he detected two variants of the latter species, characterized by frequent reduplication of the pyrenoid. This character, however, was not sustained in the progeny after sexual reproduction, and may have been attributable to cytoplasmic disturbance.

Moewus (1940*b*) reported that, by subjecting cells of *C. eugametos* to treatments of heat shock, appreciable numbers of mutant types could be obtained. Thus when cell suspensions were heated to 75° for 15 min., about 25 % of the cells were killed, and among the survivors each of certain loci concerned with sexual behaviour exhibited a mutation frequency of *c.* 0·2–0·3 %. Vegetative cells of *C. moewusii* do not survive heating to 60° for 30 sec., and no attempt was made by the present author to induce mutations in this species by heat-shock.

Of several possible mutagenic agents considered, ultraviolet was deemed the most suitable, for the following reasons: (1) The use of radiation rather than chemical mutagens obviates the necessity of subsequent washing and the dangers of persistent contamination. (2) There is good evidence that, in most organisms studied, ultraviolet effects are mainly 'point' mutations, while X-rays tend predominantly to induce gross chromosome damage (Lea, 1947). Changes of the latter sort were not desired in these preliminary investigations, since they might complicate genetic analyses. (3) An ultraviolet lamp is a convenient laboratory tool, easily handled, and occupying small space.

METHODS

Cultures were all grown in continuous light from batteries of 'daylight' fluorescent tubes, at an intensity of *c.* 470 f.c. and at a constant temperature of 23°. The media employed are given in Table 1. Further details of general methods employed in these investigations are published elsewhere (Lewin, R. A., 1952*a*).

Detection of mutant types: preliminary considerations

A Westinghouse 'Sterilamp' (model WL-782-30) was employed: the lamp was turned on 15–30 min. before use.

For all experiments in mutagenesis, in which cell aggregates are obviously undesirable, free-cell suspensions were used from young cultures grown in media of pH 7·0 or somewhat higher. In more acid media, the cells tend to form palmelloid colonies, in which most mutants would be overgrown by the associated parental-type cells.

A modification of the 'layering' technique evolved by Lederberg & Tatum (1946) for the detection of mutants was initially employed. Although in two extensive experiments a total of fifteen mutants was obtained (among them M. 151—see p. 246), use of this method was discontinued for the following reasons: (1) Irradiation in a liquid medium results in a dosage per cell which is statistically constant, but not individually uniform. Some cells may by chance receive higher dosages, while others, perhaps in a backwater of the vessel and shielded on one side by the glass wall, escape with a relatively light dose. The latter will be well represented among the surviving cells which give rise to colonies. (2) The incorporation of *C. moewusii* into liquid agar necessitates some care, since the cells are killed at temperatures approaching that at which the medium solidifies. All vegetative cells are killed by exposure to 48° for 1 min. or to 46° for 1½ min. It is certain that many cells succumb at temperatures lower than these, and this sensitivity will be a function of the physiological state of the cells (and thus presumably of the ultraviolet dosage sustained). (3) The need for three or four layers of agar multiplies the chance of invasion by aerial contaminants. This is particularly serious in the case of an organism with a generation time of several hours, where 10 days are required for the completion of the layering process. Moreover, the removal of suspected mutant colonies from the depths of the agar involves possible contamination from neighbouring colonies which, despite the precaution of 'sandwiching', may yet invade agar/agar interfaces. (4) In this preliminary study, morphological and various other mutant types were considered, though they grow well in mineral media, and would therefore be overlooked by the adoption of this method. (5) The rate of growth of all colonies is considerably reduced by the limiting rate of diffusion of carbon dioxide down to the embedded cells.

Table 1. *Composition of culture media*

BM	Beijerinck's mineral medium	
	NH_4NO_3	0·5 g./l.
	K_2HPO_4	0·2 g./l.
	$MgSO_4 . 7H_2O$	0·2 g./l.
	$CaCl_2 . 2H_2O$	0·1 g./l.
	Trace elements (Burkholder & Nickell, 1949)	
BPM	More strongly buffered, as BM but with phosphate at triple strength—0·6 g./l.	
BA	As BM, supplemented with 0·1 g. Na acetate.	
BAT	As BA, supplemented with 0·4 g. Bacto-tryptone (Difco).	
BATY	As BAT, supplemented with 0·1 g. yeast extract (Difco).	

For these reasons, as well as for general considerations of ease and convenience, a simpler method was adopted, which actually gave better results. About 65% of the putative mutant colonies obtained by the layering technique proved on subsequent testing to be indistinguishable from the parent type—colony development having followed an initial lag probably attributable to ultraviolet damage in the non-hereditary soma. In the following method, this fraction was reduced to about 50%.

Surface irradiation

About 0·1 ml. of a dense suspension of actively growing cells was pipetted on the surface of a minimal (BM) agar plate, containing a non-inhibitory concentration of an indicator dye (see p. 237). By means of a sterilized spreader (a glass rod, so bent that a straight section 5–8 cm. in length can be applied to the surface of the agar and stroked over it without breaking the surface), the suspension was dispersed as evenly as possible over the surface, and allowed to dry. The plates were then irradiated, open, for varying periods of time (0–5 min. at 10 cm. from the 'Sterilamp'), after which the covers were replaced, and the plates illuminated in the usual way without further treatment.

At the end of 5–7 days the colonies were scanned under the dissecting microscope, and all small, rough, or otherwise distinguishable colony types picked for further testing. (Plates too crowded, having received insufficient irradiation to reduce the surface population, were discarded.) As mentioned above, about half the variants so isolated proved to remain constant in subculture.

It was of interest to know the approximate percentage of cells surviving treatment, and to relate this to the proportion of mutants detected. For this reason, control (not irradiated) plates were prepared at the same time, and estimates of the numbers of viable (colony-producing) cells or cell aggregates were obtained by colony counts in random optical fields under the dissecting microscope, with which corresponding figures obtained from treated plates could be compared.

It will be noted that, by this method, the following advantages were achieved: (1) Each cell received, within the physical limitations of the system, an identical dose of ultraviolet irradiation, which had not passed through a variable depth of aqueous cell suspension before reaching it. Cells near and partially shielded by the walls of the plate remained in this position, and high marginal survivals could be recognized and discounted if necessary. (2) The cells were at no time subjected to the unfavourable temperature of molten agar. (3) The resultant colonies were superficial and co-planar, considerably facilitating the isolation of single clones, and reducing the danger of cross-contamination. (4) Morphological colony variants could be detected. (5) The plates were only opened once between irradiation and 'picking'. (6) The diffusion path of CO_2 from atmosphere to cell was reduced to a minimum.

The rationale of this method, where biochemical mutants are concerned, depends considerably on the size of the organism. The cell volume of *C. moewusii* is approximately 100 times that of a cell of *Bacterium coli*, for which the layering technique was evolved. A cell, grown in a favourable medium and irradiated without special preliminary starvation or washing, may carry with it, in its cytoplasm or adsorbed on the wall, many times the minimum requirements of such nutrilites as it may require for growth. If the genetic ability to synthesize one such nutrilite is lost, growth ceases in the absence of an exogenous supply after a few cell generations. In the case of a bacterium, the resulting colony may be invisible under low magnification: in Chlamydomonas, however, it has proved easy to observe, remove, and subculture colonies estimated to contain 32–64 cells. Among colonies of this type, it may be expected—and in two cases (M. 336, M. 701) it was actually found—that nutritionally deficient types might be picked up.

Since well-characterized mutants might arise, differing from WT in their pH relations (e.g. due to an inability to utilize the ammonium ion as a nitrogen source), an indicator dye was incorporated into the medium. Preliminary experiments had shown that 10 mg./l. of bromthymol blue, or 40 mg./l. of neutral red, approached the concentrations at which growth was inhibited, though these dilutions had no appreciable effect. Actually, the result achieved was somewhat different from that originally anticipated. Local pH changes were not made apparent in the medium. On the other hand, colonies in which for some reason the cells were dying could readily be distinguished by the fact that the dead cells accumulated the dye to an appreciable degree. Thus M. 255, the growth rate of which does not differ from that of WT, was revealed by its greater sensitivity to low pH. As the acidity of the medium (BM) rose with growth of the colonies, cells of this mutant died and took up the bromthymol blue (used in this case), so that the colony appeared orange-yellow, whereas its neighbours remained green. In most of such colonies there remained a sufficient number of viable cells to survive subculture (e.g. in one experiment, all but ten out of forty-six colonies containing dying cells were successfully transferred to complete medium and grown up).

Somewhat better results were obtained with neutral red than with bromthymol blue plates (see Table 2).

Table 2. *Results of ultraviolet surface irradiation method for the detection of mutants*

(WT+, 10 cm. from ultraviolet source.)

Period of irradiation (min.)	Neutral red (40 mg./l.)			Bromthymol blue (10 mg./l.)		
	Cols. picked	Mutants	%	Cols. picked	Mutants	%
2	21	8	38	—	—	—
2½	31	17	55	132	35	27
3	14	9	64	15	7	47
3½	—	—	—	4	3	75

Screening of putative mutants

Colonies which were suspected of representing mutant types were assigned a serial number (M. 000), transferred to agar slants of a complete medium, smeared over the surface, and grown up in the light. Since, to many mutants of Chlorella, light proved to be lethal (Granick, S. Private communication), it might have been preferable, if possible, to grow these cultures in darkness. The possibility may also be envisaged that, in the illumination following ultraviolet irradiation, some mutations may be reversed and thereby lost; or, conversely, that a lethal effect associated with a mutation might be alleviated, thus enabling the mutant to survive. Such theoretical considerations must remain closed to practical investigations until the problem of obligate autotrophy in *C. moewusii* is solved.

The exact composition of the 'complete' medium was varied in the earlier series, but later only BATY was used. The advantages of using a solid, rather than a liquid, medium at this stage were twofold: (1) The slope could be conveniently handled and stored without further transfer. (2) Cells to be used as inoculum in biochemical studies could be scraped from the surface with a minimum of associated nutrilites as 'carry-over'.

A suspension of each such culture was then made in 0·1 ml. of sterile water, and from this tube loopfuls were spread on agar test media (Perkins, 1949). Three media were employed—BPM, BA, and BATY. The use of BA in addition to a minimal (i.e. purely mineral), and a complete medium, served to distinguish at once non-photosynthetic mutants from those with other growth-factor requirements, and, in the case of the latter, provided a check for the growth on minimal.

Eleven clones were tested on each set of three test plates, using a standard pattern grid on which the plates were set before inoculation. The plates were oriented by a scratched recognition mark at the edge, and a key to the positions recorded in the protocol. On each plate, the twelfth place was occupied by a control, of the parental type which had been subjected to irradiation.

After growth had proceeded for 5–7 days, the plates were examined, and any types deviant from the parent stock on any medium were marked for further investigation. Macroscopic comparison of growth and colony form was invariably supplemented by microscopic examination of cells from the 'complete' plate, and in many cases also from the other media. In this way a number of morphological, as well as some biochemical, mutants were detected.

Finally, a transfer was made from the minimal plate into a tube of liquid BPM, in order to expose any biochemically deficient mutants which might have grown using the traces of nutrient carried over from the original 'complete' slope, or present in the agar. Several non-swimming mutants were also revealed in this way.

TYPES OF MUTANTS OBTAINED

Over half of the colonies tested behaved on subsequent transfer like the parent stocks. Among the remainder, by far the most numerous were the slow-growing and palmelloid types. These all grew in minimal media to some extent, and rather better in 'complete': but since WT cultures behaved in the same way, this provided no indication of biochemical deficiency, or of changed specific requirements. Thirty-four of these were further tested in seven different (liquid) media, consisting of minimal (BM); BM supplemented by one of the following: acetate, tryptone, yeast extract, nucleic acid hydrolysate, casein hydrolysate; and 'complete'; but no specific nutritional responses were observed. Ten mutants were tested for a requirement of unknown substances diffusing from WT cells, by streaking a suspension of WT on pour plates densely seeded with the mutant in question: no halo of satellite colonies was observed in any instance.

A few possible mutants failed to survive, for various reasons, before they could be examined microscopically and culturally. The classification of the remainder could not be entirely objective, owing to the range of variability and of characters considered, so the numbers within the classes given in Table 3 should not be regarded as more than indicative of the types of mutant *sensu lato* obtained in the course of this survey.

Certain mutants will be described in some further detail below.

Table 3. *Summary of mutant types*

Character		No. of isolates
Slow-growing (not characterized further)		65
Palmella with some motile cells	51	67
Palmella without motile cells	16	
Strong tendency to misdivisions*		10
Paralysed, etc.*		7
Pale†		6
Intracellular volutin abundant*		5
Cell form variants*		3
Flagellaless*		3
Vitamin-dependent*		2
Non-autotrophic*		1
Slight differences in colony form		25
	Total	194

* Examples of these types are dealt with separately in the following sections.

† No marked colour variants—e.g. yellow, brown or white—were found. M. 546 cells are appreciably paler than those of M. 236, from which it was derived, but in a magnesia-column chromatogram of an acetone extract no significant difference between the two types could be detected.

Mutants exhibiting cell misdivisions

One may occasionally find in WT cultures twinned cells, evidence of incompletely effected divisions. In old cultures, or in the presence of subinhibitory concentrations of various substances in the medium, such forms may be not uncommon. They have been recorded in various species of Chlamydomonas and in related genera. It is probable that many reported cases of 'gamete fusion', in which the process has been described as being initiated at the posterior ends of the cells (Mitra, 1950), are to be attributed to faulty observations of such misdivision forms (as pointed out by Lerche, 1937).

In the case of such mutants as M. 470, an internal physiological upset results in the frequent occurrence of 'Siamese twins' and other abnormalities even in young cultures; it is always associated with a reduced growth rate (Pl. 1, figs. 2, and 4–6).

M. 470(+). *Twinning*

Origin. WT+ : ultraviolet, 10 cm., 2·5 min. (surface, BM bromthymol blue agar).

This mutant was originally observed to grow less rapidly than WT, and to produce somewhat less wet colonies on BA. Microscopic examination revealed the presence of a high proportion of misdivided cells (e.g. 5–10% in young liquid cultures). Apparently longitudinal division, proceeding posteriorly from the papillar region, is frequently arrested before completion. Pairs may be found attached by a narrow bridge between the posterior ends (Pl. 1, figs. 2, 5): pairs with a wider bridge, or an extensive region of coalescence: 'cells' with two pyrenoids, two nuclei, and two papillae separated by a notch of variable depth: and, very rarely, a cell with partially fused papillae and three or four flagella as the only external indications of its double nature (Pl. 1, fig. 3). Often three or four cells may remain together, attached by basal processes: such pseudo-colonies resemble a small Pandorina.

Still more grotesque forms may be found, in which a 'cell' may bear four or more processes of irregular dimensions, each bearing a papilla and a pair of apparently normal flagella. Such monsters presumably arise from a series of consecutive misdivisions.

It was found that this mutant is more sensitive to high concentrations of sodium citrate than is WT, the limiting concentrations for maximum growth being about 0·003 and 0·01 M, respectively. Liquid media (BA) containing 0·03 M citrate permit growth, in palmellar form, of WT, while almost completely suppressing that of M. 470.

It appeared possible that the action of the citrate might be that of a chelating agent, binding some metal for which M. 470 has a high requirement. However, a threefold increase of the trace element mixture used throughout these experiments in no way alleviated citrate inhibition. Furthermore, M. 470 responds to various levels of ethylenediamine tetra-acetate (a strong chelating agent—cf. Hutner & Provasoli, 1951) in the same way as does WT. The phenomenon of citrate inhibition thus remains unexplained.

M. 470 has been used in mating experiments to introduce the character for twinning (*t*), which behaves as a single gene mutation. Citrate inhibition does not remain associated with it, and the genetics of this incidental feature have not been followed up.

M. 202(+). *Monstrous*

Origin. WT+: ultraviolet, 10 cm., 2 min. (surface, BM neutral red agar).

Although the growth rate of this form is not very much lower than that of WT, some serious disorganization is apparent from the form of the cells. Colonies on agar are dry and rough in appearance: in liquid the growth is flocculent, accumulating on the bottom. Most of the cells are grossly misshapen, occasional inflated spheroid forms reaching 50μ. diam., and the lobed, misdivided thalli may contain a multiplicity of nuclei and pyrenoids (Pl. 1, figs. 4, 6). They are as a rule devoid of flagella (occasionally one or two are borne, these generally being shorter than in WT), and, when motile, the mode of swimming is slow and irregular.

No nutritional requirement or pronounced influence of nutrients in the medium has been observed, other than those also exhibited by WT.

Paralysed mutants

Among 132 putative mutants arising from irradiation of WT+ cells, three (M. 269, M. 375 and M. 475) were identified as being in some way impaired in their flagellar activity: among a further ninety-four obtained from M. 255 ('volutin'), three more (M. 668, M. 700 and M. 701) were found. These, with a mutant (M. 236) which arose from WT+ in an earlier experiment, appear to fall into two groups: (1) Paralysed—in which the flagella of the majority of cells remain extended without beating, or occasionally beat at about 1–2 beats/sec. (compared with about 12 for WT cells). The tips of the flagella—the distal third—twitch sporadically. The autonomous creeping of flagella (Lewin, R. A., 1952*c*) is readily apparent in this type, the cells of which may be drawn forward at about 2μ./sec. (M. 236, M. 269, M. 475, M. 668 and M. 701). (2) 'Lazy'—in which the flagella of most cells beat slowly and irregularly, without effectively propelling the cells through the water (M. 375 and M. 700).

In cultures of both categories, especially in those more than a week old, there are found swimming cells, progressing in a wobbling fashion at a rate slower than that typical of WT. The flagellar beat may be of the order of 4–8 beats/sec., and the rate of progress (*c.* 25μ./sec.) about half that of the normal cells (*c.* 60μ./sec.). Liquid cultures can be at once distinguished from those of fully active cells, since cells of paralysed and 'lazy', as well as flagella-less and palmelloid, stocks form bottom deposits, whereas WT and most other mutants produce surface films.

As far as has been determined, these mutant flagellar characters are dependent on single genes: in the cases of M. 236, M. 375 and M. 701 these are not allelic, and appear unlinked (Lewin, R. A., 1952*b*). It may ultimately be possible to garner biochemical information on such mutants which may throw light on the mechanism of flagellar movement as a general phenomenon. So far, unfortunately, no means has been found by which the condition of paralysis can be 'cured' in genetically paralysed cultures.

The origins of the various mutants affecting flagellar characters are summarized in Table 4.

M. 236(+). *Paralysed*

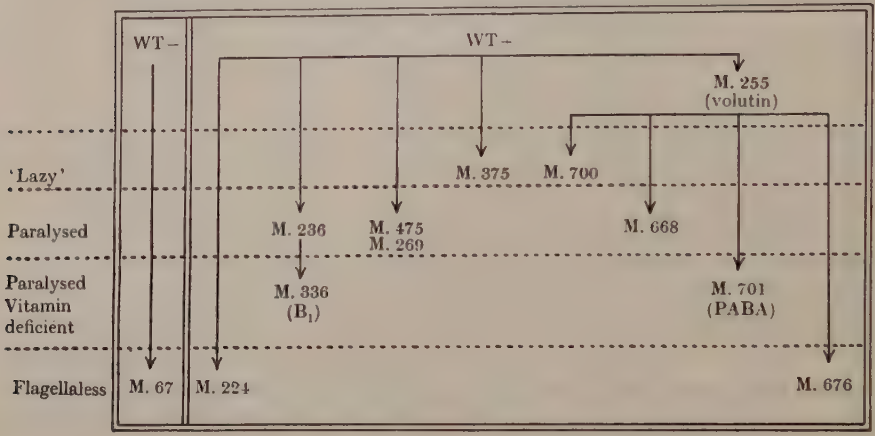
Origin: WT+: ultraviolet, 10 cm., 2·5 min. (surface, BM neutral red agar).

This was the first of the series of paralysed mutants to be observed and investigated: further mutants (M. 275–373, M. 406–437 and M. 521–600) were obtained by irradiation of M. 236 cultures.

All attempts to stimulate normal flagellar activity were abortive: such substances as adenosine triphosphate and acetylcholine, glutathione, glucose, hydrogen peroxide, hypertonic sodium chloride, weak acids and alkalis, etc. were without effect in short-term (10–15 min.) tests. A wide range of dilutions of various WT cell hydrolysates, of glucose (0·125–2·0 %) and of an aqueous extract of *Crocus sativa* stigmas (cf. Moewus, 1943) likewise produced no modification of the paralysis during growth.

This character serves in ready fashion to distinguish the copulants + from − during pairing (Lewin, R. A., 1950*a*). When the − partner alone is paralysed, the pairs swim in the fashion normal in WT + × WT − matings, since the activity of the + partner is unimpaired. When the reciprocal mating is made, the pair does not swim, though the − partner before copulation was capable of normal activity. When both partners are paralysed, mating is of course considerably hampered and copulant pairs are sparse, but these again are found to be non-swimming.

Table 4. *Pedigrees of mutants with flagellar aberrations*



Among the mutants derived by further irradiation of M. 236, M. 336 proved to require aneurin for optimal growth. The latter strain, bearing thus a biochemical marker additional to the gene for paralysis, was used in preference to M. 236 for genetic study.

M. 375(+). *'Lazy'*

Origin: WT + : ultraviolet, 10 cm., 2·5 min. (surface, BM bromthymol blue agar).

Like M. 236, the cells of this mutant accumulate at the bottom in liquid cultures, though in old cultures (e.g. 2 weeks) there is a tendency for the cells to swim through the medium. Their motion is then slow and trembling, however, and unlike that of WT cells.

M. 375 and M. 700 were distinguished from the other mutants of the paralysis series by the greater activity of the non-swimming cells. These perform spasmodic and usually unilateral movements of the flagella, which, beating slowly and asynchronously, fail effectively to propel the cells. Despite this difference of behaviour in young cultures, it is not always possible to distinguish clearly between paralysed and 'lazy' stocks (e.g. in segregates of a cross involving both types).

Volutin-rich mutants

M. 255(+). *Volutin*

Origin. WT+: ultraviolet, 10 cm., 3 min. (surface, BM bromthymol blue agar).

This mutant, resembling WT in colony form and growth rate on mineral as well as complete media, was revealed by its greater sensitivity to a lowered pH, as a result of which cultures in unbuffered media exhibit a greater tendency to early bleaching. In one experiment, the final pH after 10 days' growth in BM was 3·92 (average of five replicates, ranging from 3·83 to 4·00), while that of the WT control was 3·23 (five replicates, 3·13–3·36). Bleaching occurs regularly in BM plates in which the medium is only poured to a depth of 3 mm.: where acidification is slower in deeper media of greater volume, the cultures remain green considerably longer.

Microscopically the cells are readily distinguished by the presence of large, refractile globules of 'volutin', which tend to distort the form of many of the smaller cells (Pl. 1, figs. 7, 8). These bodies do not stain with iodine, or with Sudan III or osmic acid, but readily take up neutral red, and presumably correspond with the 'vacuome' described (in Chlamydomonas, etc.) by Hall & Nigrelli (1931). They exhibit metachromasy when stained with methylene blue; i.e. light transmitted by lightly stained globules tends to be shifted towards the longer wavelengths, so that these bodies take on a lilac or magenta colour.

It was suspected that these 'metachromatic vacuoles' (Hollande, 1942) might consist of metaphosphate (cf. Wiame, 1949). Supporting evidence for this was obtained from a series of cultures in modified BA, in which the amount of phosphate was varied. When phosphate was 'omitted' (traces introduced with the other constituents of the medium and with the inoculum permitting slight growth), no volutin was apparent in either WT or M. 255 cells. At 0·05 g. K_2HPO_4/l., small metachromatic granules appeared in the cytoplasm of most cells in the WT culture, while in M. 255 these were often accompanied or replaced by one or two large globules. In 0·15 g. K_2HPO_4/l. or more, all cells of the mutant contained large globules, ranging up to 5μ. diam. The WT cells contained small granules not exceeding 1·5μ. diam.; they appeared in some numbers, about twenty to thirty typically being clustered in the region of the nucleus (cf. Strehlow, 1928–9). In older cultures of WT, however, they may be larger (cf. Czurda, 1935, fig. 6*c*).

This character of M. 255—the production of large volutin globules—proved to be a convenient marker. When cells so labelled were mated with normal gametes, it was possible to distinguish the partners, *plus* from *minus*, and in this way to elucidate differences in their behaviour (Lewin, R. A., 1950*a*). For many hours, up to the time of nuclear fusion and zygote formation, the respective cytoplasms of the two copulants could be recognized in this way.

By irradiation of M. 255 cultures, a series of mutants was obtained, including a range of variants similar to those obtained from WT cells, but marked with the volutin character.

From matings with WT, this character was introduced into cells of *minus* mating-type.

Zygospores homozygous for this character developed regularly and were normal in appearance.

M. 389(+). *Volutin*

Origin. WT+: ultraviolet, 10 cm., 2·5 min. (surface, BM bromthymol blue agar).

In all essential respects this mutant resembles M. 255. When M. 389 was crossed with Z. 717/2, bearing the volutin gene from M. 255, zygotes were produced which proved to give extremely low and irregular germination after the dark treatment found successful for WT crosses (Lewin, R. A., 1949). This may be attributed to the same alteration in metabolism as that responsible for the formation of large volutin globules in the haplophase. On analysis of one zygote obtained from this cross, a clone without the volutin character was obtained, indicating that different loci are involved.

Cell-form variants

M. 478. *W*(+). *Spherical*

Origin. WT+: ultraviolet, 10 cm., 2·5 min. (surface, BM bromthymol blue agar).

This differs from WT not only in its slower growth, but also in cell form, which is characteristically spherical. The cells are small, usually only 4–6μ. diam., and progress in a slow, jerky fashion: in liquid culture they accumulate on the floor of the vessel, comparatively little surface growth being formed.

Flagellaless mutants

M. 67(−). *Flagellaless*

Origin. WT−: ultraviolet, 15 cm., 5 min. (layering).

The growth rate of this mutant approaches that of WT in mineral media. On agar, the colonies appear less smoothly lenticular: in liquid, the cells remain at the bottom of the vessel. The cells are completely devoid of flagella at all stages and in all media tested. Specifically it may be noted that no effect was produced either by glucose or by aqueous extracts of *Crocus sativa* stigmas, used in a wide range of concentrations (cf. Moewus, 1943). In other ways there appear to be no marked differences from WT, though there is a stronger tendency for the formation of palmellar clumps. Gamete-like cells—identified by their size and shape and by their mode of development—are produced: lacking flagella, however, they are incapable of copulating.

Vitamin-dependent mutants (Lewin, R. A., 1950*b*)

M. 701(+). *PABA-less* (*paralysed*)

Origin. M. 255: ultraviolet, 10 cm., 3 min. (surface, BM neutral red agar).

The stock from which this mutant was obtained had already a genetic marker—'volutin'. When M. 701 was isolated, it was found to differ from

M. 255 in two features: (1) paralysed flagella, like those of M. 236, (2) a partial requirement for exogenous *p*-aminobenzoic acid (PABA), in the absence of which growth is considerably retarded. At least three possible hypotheses were entertained to account for this double change: (*a*) the two altered phenomena might be manifestations of a single mutated gene; (*b*) a small deletion of a chromosome, bearing genes affecting motility and vitamin synthesis, may have occurred; (*c*) two independent mutations had taken place simultaneously.

But augmenting the PABA in the medium did not induce motility: and motile cells of M. 255 were not paralysed by any concentration of sulphanilamide tested, which might be expected to interfere with PABA metabolism. These facts seemed to militate against possibility (*a*) above.

The matter was clinched by mating experiments in which PABA-requirement segregated in normal fashion from paralysis, both behaving as single genes, apparently unlinked (Lewin, R. A., 1952*b*). The simultaneous occurrence of these two mutations may be considered less surprising when it is realized that eight mutants of the paralysed series appeared among 226 putatives tested from surface irradiations, of which eighty-one maintained, in subculture, recognizable differences from the parent stock. There might then be a 10% expectation of a biochemical deficiency mutation arising at the same time as an impairment of the flagellar mechanism.

Like the PABA-requiring strain of Neurospora investigated by Tatum & Beadle (1942), M. 701 was not stimulated by benzoic acid, but growth could be promoted by aniline. 100 mg./l. completely inhibited growth of this mutant; in 25 mg./l. the growth rate approached the maximum obtainable in PABA media; while there was some response to concentrations as low as 1 mg./l. of aniline. The fact that the relative efficiency of aniline was of the order of 1:100 (cf. the value 1:14,000, given by Tatum & Beadle for the Neurospora mutant) indicated that aniline probably does not lie on the direct path of PABA synthesis. Sulphanilamide inhibition of WT cultures was reversed by PABA but not by aniline—further evidence against the possibility that PABA synthesis normally passes through aniline as an intermediate.

M. 336(+). *Thiamin-less* (*paralysed*)

Origin. M. 236: ultraviolet, 10 cm., 2·5 min. (surface, BM bromthymol blue agar).

This mutant arose from a paralysed stock, and further differed from WT in a biochemical feature. Tested in BATY and in BA, M. 336 was found to grow extremely poorly in the latter medium. It responded particularly well to additions of yeast extract and of a synthetic vitamin mixture: further tests in BA to which single vitamins had been added showed that such a stimulation could be produced by aneurin (thiamin) alone.

In media supplemented by aneurin, the growth rate approaches that of M. 236; in the absence of this factor, growth is very slow, though in both BA and BPM the cells multiply eventually. (Cf. two out of the four aneurin-less mutants of Neurospora described by Tatum & Bell (1946), in which the requirement was found not to be absolute.) At 50 mg. aneurin/l. there is some inhibition, growth proceeding maximally at levels between 0·001 and 0·1 mg./l., and an appreciable response being obtained to concentrations as low as 0·0001 mg./l. (i.e. 4×10^{-10} M). Lwoff (1947) reported that the lower threshold for aneurin in *Polytomella caeca* is of the order of 2×10^{-10} M.

The vitamin requirement for such a mutant might be satisfied by the free aneurin present in such natural habitats as lake water, where it may reach 0·001 mg./l. (Hutchinson, 1943) or in soil, where it must be considerably higher. Both van Overbeek (1940) and Ondratschek (1940) reported a marked stimulation of Sphaerella—'wild' species—by aneurin, while the latter author published an account of similar phenomena in six Chlamydomonas species. (This work of Ondratschek is open to considerable criticism, however: see Lwoff, 1947.) Thus a mutation of this sort might be under only slight adverse selection in nature.

The thiazole and pyrimidine moieties of aneurin (4-methyl-5-*beta*-hydroxyethyl thiazole: 2-methyl-5-ethoxymethyl-6-aminopyrimidine) were tested for their ability to replace the complete thiamin molecule. The results are summarized in Table 5.

Table 5. *Response of M. 336 in BA medium to moieties of aneurin*

Supplement	Growth
Control	−
0·1 mg./l. thiazole	−
0·1 mg./l. pyrimidine	+
Thiazole + pyrimidine	+
0·2 mg./l. aneurin	+

These results indicate that the organism retains unimpaired the abilities to synthesize thiazole and to couple it with pyrimidine, but its synthetic ability towards pyrimidine is reduced. It may be noted that *Polytoma caeca* requires additions to the culture medium of both constituents of the aneurin molecule, while *Polytomella ocellatum* requires the thiazole moiety alone. M. 336, in its synthetic disability, resembles *Euglena gracilis* (Lwoff, 1947).

Mutant with impaired photosynthetic system

M. 151(−). *Non-autotrophic*

Origin. WT−: ultraviolet, 15 cm., 2 min. (layering).

This strain was found to be incapable of appreciable growth in mineral liquid media, where it produces small granules of palmella which only become visible to the naked eye 3–4 weeks after inoculation. On media containing an organic carbon-source, however, rates of growth of M. 151 approach those of WT.

A number of compounds were tested for their ability to support growth. Among carboxylic acids (0·007 M, adjusted in BM to pH 7·1), citrate provides a very suitable source of organic carbon, while good growth can also be obtained with fumarate, succinate, pyruvate or malate: growth with acetate is poorer. Glucose and glycerol also provide suitable carbon sources for growth.

This mutant has been shown to exhibit the Hill reaction (Lewin, J. C., unpubl.), and to carry out photosynthesis when supplied with 5% CO_2 (Frenkel, A., 1949, private communication). Further studies on its metabolism will be published elsewhere.

Cultures of M. 151 were found to grow in mineral media supplemented with yeast extract (0·1%), but this effect could not be reproduced by the addition of pure vitamins, either singly or in combination, and it was deduced that the extract merely provided a source of organic carbon.

The pigments of M. 151 and WT cells were compared on a sucrose chromatographic column, using petrol ether as solvent. No significant differences were observed.

For the production of active gamete suspensions, it was found that cultures grown on acetate (BA) were inferior to those on citrate media, and the best results were obtained when both sodium acetate and sodium citrate (0·1% of each) were incorporated into the medium.

M. 151 has been carried through a number of crosses, in which the mutant character (*n*) has behaved as a single Mendelian factor. There is a considerable reduction of viability in segregates bearing this gene, rendering the genetic data for this character very incomplete (Lewin, R. A., 1952*b*).

As a result of crosses, the character (*n*) has been transferred to stocks of *plus* mating-type, and zygotes homozygous for *n* have been obtained. They require a longer period of illumination for maturation, but ultimately develop on mineral agar, and then appear indistinguishable in size and sculpturing from WT zygospores. This is of some interest, since a 30-fold increase in bulk is thus effected by the diploid cell in the absence of an organic carbon-source; this corresponds to about five generations of vegetative cells in the haploid condition.

The foregoing data are part of a dissertation presented to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. The work was carried out during tenure of Sheffield and H. D. Hooker Fellowships between 1948 and 1950. The author is particularly indebted to S. E. Reaume for many valuable ideas and criticisms in this phase of the investigation; and to various members of the Osborn Botanical Laboratory, to S. H. Hutner and L. Provasoli of the Haskins Laboratories, and to J. C. Lewin.

The samples of thiazole and pyrimidine were obtained through the kindness of Dr J. C. Woodruff of Merck and Co.

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EXPLANATION OF PLATE

(Magnification: figs. 1–6, × 1000; figs. 7, 8, × 1500.)

Fig. 1. Normal, wild-type cell. (Osmic acid; gentian violet.)
Fig. 2. Twinned cells (M. 470). (Osmic acid; gentian violet.)
Fig. 3. Cell with four flagella (M. 470). (Osmic acid; gentian violet.)
Fig. 4. Lobed cell masses (M. 202). (Osmic acid; gentian violet.)
Fig. 5. Twinned cells (M. 470). (Acid hydrolysis; gentian violet.)
Fig. 6. Lobed cell masses (M. 202). (Acid hydrolysis; gentian violet.)
Figs. 7, 8. Cells with large volutin globules (M. 255). (Methylene blue.)

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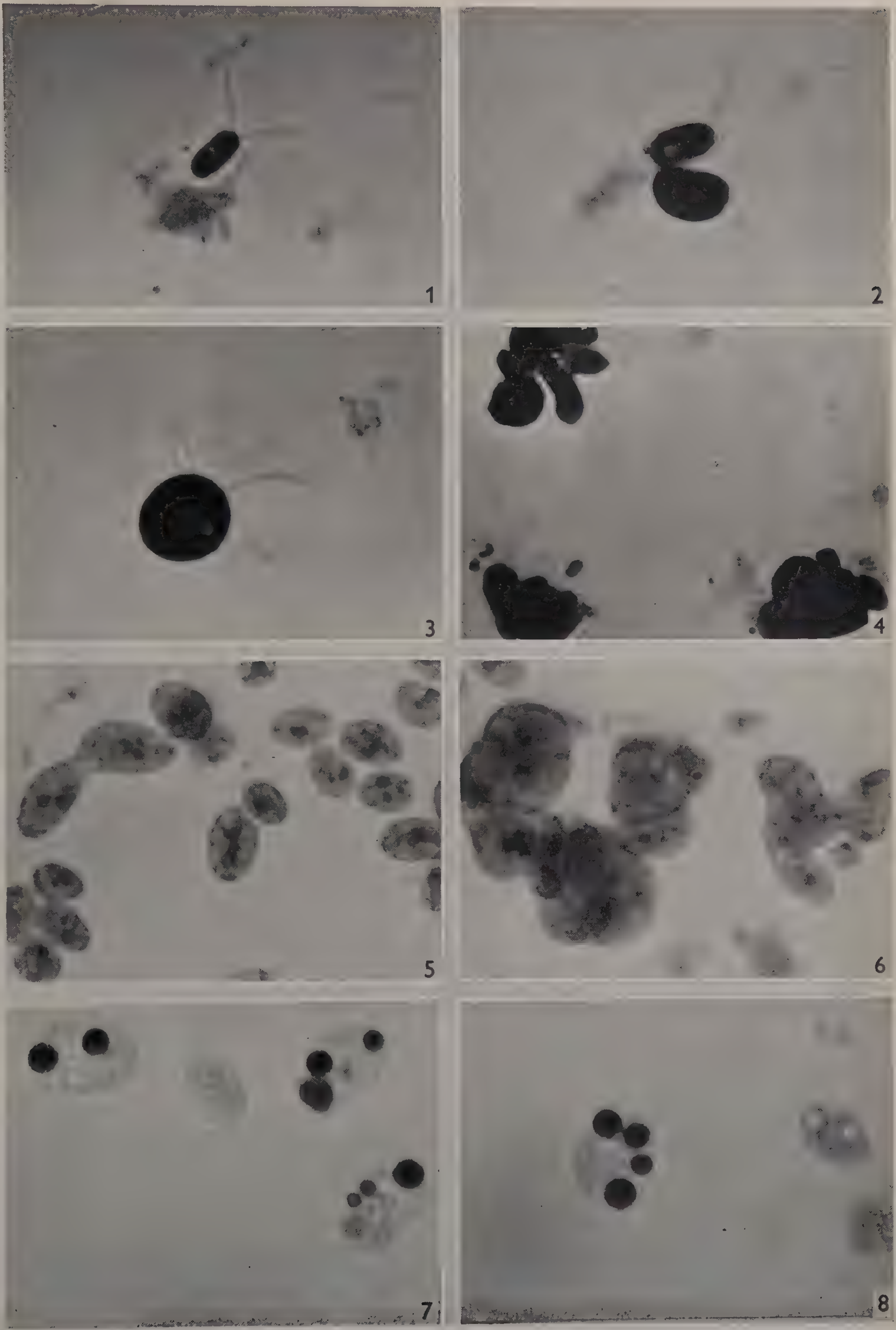

R. A. Lewin—Ultraviolet mutations in *Chlamydomonas moewusii*. Plate 1

LEWIN, R. A. (1952). *J. gen. Microbiol.* 6, 249–250.

The Primary Zygote Membrane in *Chlamydomonas moewusii*

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SUMMARY: In *Chlamydomonas moewusii* the primary zygote membrane (PZM) is formed at the initiation of gamete fusion. The PZM of *C. moewusii* does not appear to be composed of cellulose or pectic material; it stains readily with Schiff's reagent which suggests the presence of free aldehydic groups. Several recently isolated strains of *Chlamydomonas* show this reaction which is presumed to be common to the genus.

The fusion of gametes in *Chlamydomonas paupera* is followed by the formation of a 'primary zygote membrane' (PZM) (Pascher, 1931–2). Within this membrane the thick-walled zygospore is formed, and the PZM is ultimately shed. A similar series of events has been described for *C. braunii* (Goroschankin, 1890), *Chlorogonium oogamum* (Pascher, 1931), *Gonium pectorale* (Schreiber, 1925) and *Sphaeroplea* spp. (Fritsch, 1929): it is probably of widespread occurrence, at least among the Volvocales.

In *Chlamydomonas moewusii* Gerloff, deposition of the PZM commences at the initiation of gamete fusion, before the nuclei have come together. The gametes of this species resemble the vegetative cells, and are each enclosed in a thin cell-wall; but these walls are shed during cell fusion, remaining attached to the PZM as delicate hyaline vesicles. In conditions of constant artificial illumination the formation of the true zygospore wall within the PZM begins within 24 hr., and after 48 hr. has achieved its final form, being considerably thickened and sculptured with a coarse reticulum. Since these changes are accompanied by considerable enlargement of the zygospore, the PZM is usually ruptured in the process, and may be shed and completely freed from the spore (Pl. 1, figs. 3, 4).

The walls of the vegetative cell of the gamete and of the mature zygospore appear birefringent under crossed Nicols, and are presumably cellulosic in nature. The PZM, on the other hand, does not exhibit this feature, nor does it stain with ruthenium red, indicating that it is composed of neither cellulose nor pectic material. Goroschankin (1890) reported that the PZM in *C. braunii* stained blue with Schultze's reagent, chloriodide of zinc, differing in this respect from the walls of vegetative cells and zygospores. This was not found to be the case in *C. moewusii*. The mature zygospore wall of *C. moewusii* is extremely impermeable, but the PZM permits the penetration of dyes (e.g. gentian violet): these observations are in agreement with those of Schreiber (1925) on *Gonium pectorale*. Unlike the walls of vegetative cells and zygospores, the PZM of *C. moewusii* stains readily within a few minutes when treated, without prior hydrolysis, with Schiff's reagent (fuchsin decolorized with SO_2)

(Pl. 1, figs. 1, 2). This suggests the presence of free aldehydic groups within the membrane. There is some confirmation of this in the fact that the colour is immediately bleached by alkali and reversibly restored by acid, which is said to be indicative of aldehydic substances (Oster & Mulinos, 1944).

Sexual strains of three homothallic and three heterothallic species of *Chlamydomonas* have recently been isolated, from a ditch in Long Island, N.Y., from a greenhouse in New Haven, Conn., and from soil samples collected in Arizona (Lewin, 1951). In every case these species resembled *C. moewusii* in that only the PZM became stained on treatment with Schiff's reagent; so it may be presumed that this reaction is common to members of the genus.

Prát (1947), in a study of the reaction of algal cell walls to Schiff's reagent, tested vegetative cells of fifteen species of *Chlamydomonas*, as well as members of other genera of the Volvocales, and in no case observed recoloration of the dye. He found, however, that whereas the normal vegetative cell walls of *Spirogyra* spp. (Conjugales) do not stain with this reagent, the walls of copulating cells, especially in the region of the copulation tube, stain deeply within a few seconds. This difference between walls of vegetative cells and those of cells undergoing sexual fusion is thus closely parallel with that described above for *Chlamydomonas*.

No suggestion is put forward as to the significance of this reaction.

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EXPLANATION OF PLATE

Fig. 1. Young zygote among vegetative cells. (Schiff's base: × 1000.)

Fig. 2. Two zygotes—one germinating, the other collapsed. (Schiff's base: × 1000.)

Fig. 3. Mature zygospores with PZM. (Unstained: × 500.)

Fig. 4. Empty zygospore wall with PZM. (Unstained: × 1000.)

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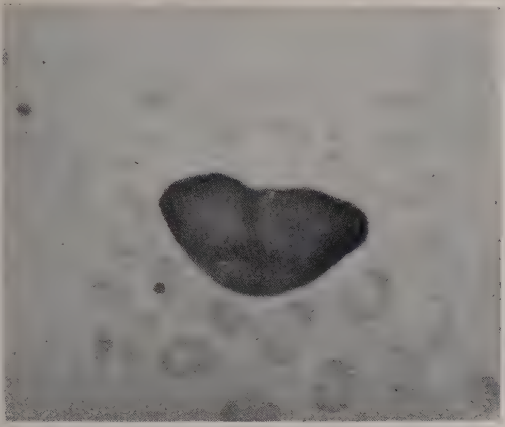

Fig. 1

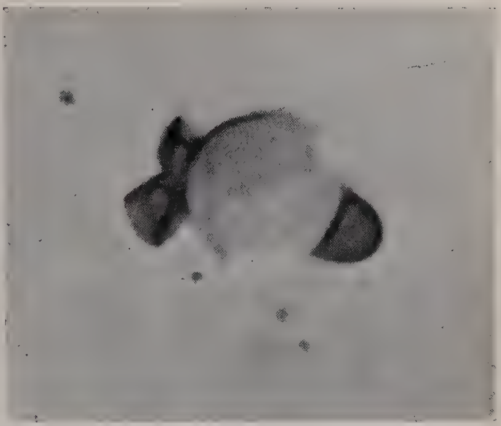

Fig. 2

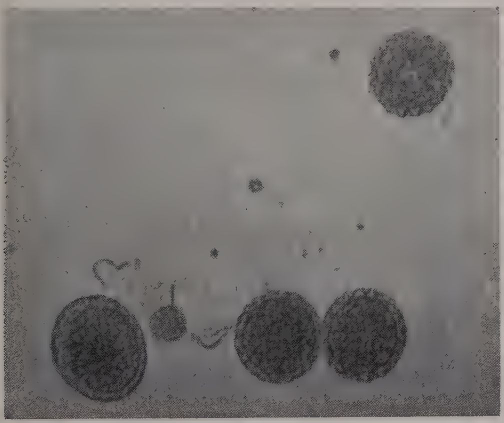

Fig. 3

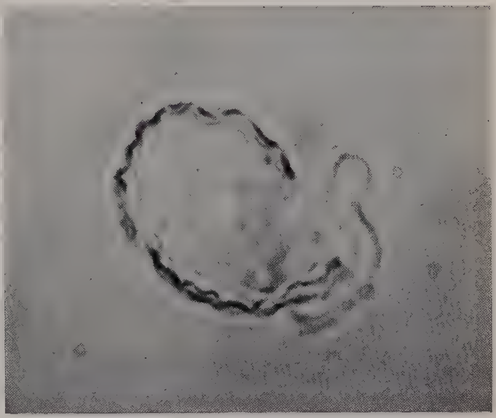

Fig. 4

R. A. LEWIN—PRIMARY ZYGOTE MEMBRANE IN *CHLAMYDOMONAS MOEWUSII*. PLATE 1

GLOVER, R. E. (1952). *J. gen. Microbiol.* 6, 251–260.

The Growth of *Mycobacterium tuberculosis* and *Mycobacterium johnei* in a Modification of Dubos Medium

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SUMMARY: Rapid multiplication of *Mycobacterium tuberculosis* can be obtained in Dubos medium modified by the addition of an iron salt and by the replacement of albumin fraction V by the supernatant fraction of 'Liquoid'-treated bovine serum.

Certain strains of *M. johnei* can be trained to grow in this medium enriched with a glycerol extract of *M. phlei*.

The success achieved in growing *Mycobacterium tuberculosis* in liquid medium (Dubos & Davis, 1946) has aroused interest in the possibility of growing *M. johnei* in this fluid or similar media, modified to contain the essential growth-promoting substances for these organisms. So far no success has been achieved (Davis & Dubos, 1948; Taylor, 1950).

The combination of Tween 80 and bovine albumin fraction V has certain disadvantages which are not completely overcome by modifications introduced by Dubos and his co-workers (Dubos, 1947; Dubos & Middlebrook, 1948; Davis & Dubos, 1948). When added to the rest of the medium, the water soluble lipids selected by Dubos acquire growth-inhibiting properties on standing for several weeks; in consequence, it is necessary to use freshly prepared solutions. In addition, fraction V bovine albumin on incubation with Tween 80 may give rise to toxic substances which are related to the lipase content of the serum (Davis & Dubos, 1948). To some extent this effect can be reduced by heating albumin solution for 30 min. at 56° to destroy most of the lipolytic activity.

In the course of experiments to find a substitute for fraction V it was found that Dubos medium without albumin, but enriched with serum treated with 'Liquoid' (Na polyanethol sulphonate; Roche Products Ltd.) was suitable for the cultivation of the tubercle bacillus and that, with certain modifications, this medium also grew *M. johnei*. As any new medium incapable of supporting the growth of the tubercle bacillus was unlikely to be of value for *M. johnei*, all trials were run in parallel with a strain of *M. tuberculosis* var. *bovis* which was regarded as an appropriate control test organism.

METHODS

Medium. The basic medium containing asparagine as a source of nitrogen was, with some minor modifications, that of Dubos (1947). Tween 80 was increased from 0·05 to 0·1 %, Vegex extract of yeast was replaced by Yeastrel (Paterson & Winmill, 1950), and 0·002 % of iron ammonium citrate was added.

In the preliminary experiments, after the addition of the Tween 80, the salt solution was distributed in 5·0 ml. amounts in McCartney bottles and autoclaved at 15 lb./sq.in. for 10 min. Sterile glucose Yeastrel solution and fraction V albumin or Liquoid-treated serum, sterilized by filtration through a Seitz EK pad, were added before use. Later it was found that better growth was obtained when the medium was sterilized by filtration. Modifications necessary for the growth of *M. johnei* will be described in the relevant sections of the paper.

Cultures. Two human strains of *M. tuberculosis*, one of which was H37Rv, two bovine strains and one avian strain were used; all were fully virulent for the appropriate laboratory animals. Eight strains of *M. johnei* were available. Cultures in Dubos medium were started by seeding a well-dispersed saline suspension of a young culture from solid medium. Subcultures were made by transferring about 0·1–5·0 ml. to fresh medium.

Estimation of growth. The density of the growth was estimated by a sensitive photoelectric densitometer similar to that described by Belyavin, Westwood, Please & Smith (1951). Standard tubes containing 1·0, 0·9, 0·8, 0·7, 0·6, 0·5, 0·4, 0·3, 0·2 and 0·1 mg./ml. of a well-dispersed culture of *M. tuberculosis* from solid media in normal saline were preserved with 0·2 % formalin. The sensitivity of the galvanometer was adjusted so that mid-scale deflexion was obtained with 0·5 mg./ml. The slight colour of Dubos medium enriched with serum does not materially alter the basic readings.

RESULTS

Properties of Liquoid-treated serum

Liquoid, first used as a powerful anti-coagulant, was subsequently found to depress the normal bactericidal properties of fresh blood and thus encourage the growth of small numbers of organisms. It has been suggested that this action may be related to an inactivation of complement (van der Holden, 1940; Stuart, 1948), to the destruction of bactericidal substances in the blood (von Haebler & Miles, 1938; Hoare, 1939), to the paralysis of leucocytes (Allgöwer, 1949), or to an effect on other growth-inhibiting substances in the blood.

To prepare the serum fraction for the replacement of albumin fraction V in Dubos medium, Liquoid was added to the serum. Suitable concentrations produced a fine precipitate; the turbidity was measured by the densitometer, and successive batches of the same sample of serum gave identical readings. Other samples, from different animals of the same species, or from the same animal at different times, often gave readings indicating a higher or lower concentration of particulate matter. These differences were also demonstrated by centrifuging the treated serum at 5000 r.p.m. for 10 min. and weighing the sediments.

When similar samples of the same serum batch were treated with decreasing amounts of Liquoid, the quantity of precipitate steadily declined. No difference was detected in samples treated with 2, 1 and 0·8 mg./ml. serum, but with less the turbidity fell until, with an amount of 0·05 mg./ml., no detectable reading was observed and only a trace of sediment was produced on centrifugation. Serum from clotted blood yielded heavier sediments than citrated or oxalated plasma, indeed many samples treated with citrate gave only a trace of precipitate with the maximum quantity of Liquoid. This suggested that calcium was essential for the formation of the precipitate. Chorine (1941) postulated that Liquoid acts as a protein precipitant; he showed that if plasma is treated with Liquoid and the precipitate removed, no further deposit was produced by the addition of more Liquoid: however, with the addition of calcium chloride, a second precipitate was formed which was also protein in nature. The need for calcium was shown in the following manner. Samples of serum and citrated plasma from the same guinea-pig were treated with 1 mg. Liquoid/ml. In the serum a heavy precipitate formed, whereas in the plasma only a slight haziness developed. The addition of $CaCl_2$ to the Liquoid plasma to make a dilution of 1/1000 resulted in a heavy precipitate which is probably produced by an interaction of some plasma constituent, the Liquoid and the calcium ions, and is not a component of the plasma deposited on an insoluble calcium-Liquoid derivative because, in the absence of plasma or serum, calcium salts do not form a precipitate with Liquoid.

The amount of precipitate was dependent on the hydrogen-ion concentration of the serum; it was small in samples at pH 7·8 or over, even in the presence of calcium, but increased when the serum was acidified. A very heavy precipitate with a nearly water-clear supernatant was produced by mixing serum, 1/1000 calcium chloride and 1/1000 glacial acetic acid in equal quantities. Such supernatants did not contain the growth-promoting factors necessary for the Tween 80 medium.

Sediment from Liquoid-treated plasma or serum was only partially soluble in water or normal saline, and it became increasingly insoluble if it was not removed as soon as possible. It was readily soluble in 1/1000 sodium carbonate.

In view of the statement by Davis & Dubos (1946) that the albumin constituent of serum is the component which protects acid-fast organisms against the toxic action of Tween 80, the sediments and supernatant fractions of Liquoid-treated serum were examined. To obtain a sediment free from the constituents of the supernatant portion, the precipitate produced by the action of 1 mg. Liquoid/ml. serum in the presence of an equal amount of calcium chloride (1/1000) was washed three times with a mixture of 10 ml. distilled water containing 1 mg./ml. Liquoid, 5 ml. of 1/1000 calcium chloride and 5 ml. of 1/1000 glacial acetic acid in which the sediment was completely insoluble. After the final washing, the sediment was dissolved in 1/1000 sodium carbonate solution and a rough analysis of the two portions gave the following results:

	Supernatant	Sediment
Euglobulin	++	+
Pseudoglobulin	±	+
Albumin	−	++

It is clear that all the albumin is concentrated in the sediment. It will be shown that the supernatant portion was active in promoting the growth of acid-fast bacilli and the sediment was valueless. It appears, therefore, that the stimulation of growth may be dependent on the euglobulin and not on the albumin. The Liquoid-treated fraction of serum added to the Tween medium is the supernatant portion after removal of the precipitate.

The growth of Mycobacterium tuberculosis *in modified Dubos medium*

In Tween medium containing fraction V albumin, a human strain such as H37Rv will produce a distinct haziness after 48 hr. at 37°. Growth increases progressively, but the maximum density is not reached before the tenth day. In Tween medium enriched with Liquoid-treated bovine serum (L.T.S.) and containing a small quantity of ferrous ammonium citrate, the organisms multiply more rapidly and the density of a 4-day culture is about equal to that of a 7-day growth in the conventional Dubos solution (Table 1).

Table 1. *Comparison of the effect of fraction V albumin and Liquoid-treated serum on the growth of* Mycobacterium tuberculosis

	Growth (mg./ml.)									
	Base + fraction V albumin					Base + Liquoid-treated serum				
	Days					Days				
	3	4	5	7	10	3	4	5	7	10
Human	0·1	0·3	0·3	0·5	0·8	0·2	0·5	0·6	0·8	1·0
Bovine	—	tr.	0·1	0·3	0·5	tr.	0·2	0·2	0·5	0·7

— = no growth; tr. = trace turbidity.

Experiments were made to find the optimum amount of Liquoid required to give the most active supernatant fluid. The proportions varied from 5 to 0·05 mg./ml., always in the presence of 1/1000 calcium chloride. The most rapid growth occurred in the range 2–0·5 mg./ml.; from this point the quantity of insoluble material steadily decreased, as shown in the previous section, and the supernatant fluids became less active. With concentrations of Liquoid in excess of 2 mg./ml., growth was delayed and partly inhibited. This was not due to an increase in the precipitate, as further additions of Liquoid to the supernatant fluid from serum treated with 2 mg./ml. failed to produce an additional precipitate. It is probable, therefore, that Liquoid in excess of 2 mg./ml. has a direct inhibitory action on growth (Table 2). The amount finally selected was 1 mg./ml.

Some latitude is permissible in the proportion of L.T.S. which can be added to the basic medium as growth of all three types of tubercle bacilli was obtained with amounts varying from 2 to 20 %; the most consistent results were obtained with 5 % and this amount was finally adopted. The sediment from Liquoid-treated serum, redissolved in weak alkali, neutralized, and added to Tween medium in the same proportion as the L.T.S., completely inhibited growth of a human strain.

Various types of serum were fractionated with Liquoid and tested for growth-promoting properties. Bovine serum gave the best results, but good

Table 2. *Growth of* Mycobacterium tuberculosis *in basic medium with Liquoid-treated bovine serum*

Liquoid (mg./ml.) added to serum	Growth (mg./ml.) after 10 days with strain		
	H	B	A
5·0	0·3	0·1	0·2
2·0	1·0	0·7	1·1
1·0	0·9	0·8	1·0
0·5	0·8	0·6	1·0
0·2	0·4	0·2	0·8
0·1	0·2	0·1	0·5
0·05	0·2	tr.	0·2
None	—	—	—

H = human; B = bovine; A = avian.
Symbols as in Table 1.

cultures were obtained with L.T.S. from horse, sheep and rabbit sera, although longer incubation was required. The difference may be due to variations in aggregation of the insoluble fraction since, even in the presence of calcium chloride, the amount of precipitate, particularly in horse sera, was less than in bovine sera.

In an article on Tween medium (Dubos & Davis, 1946), it was emphasized that no systematic investigation of the constituents of the basal medium had been attempted. Later, Dubos (1947) devised a modified solution containing ammonium sulphate which was used for determining the effect on growth of various combinations of glucose, casein, ferric chloride and Tween 80. It seemed desirable to ascertain whether variations in the amount of asparagine, glucose and Tween 80, in the presence of L.T.S., would have any material effect on the rate of growth. Table 3 clearly indicates that there is a critical level for each constituent. The optimum amounts were 0·1–0·5 % glucose, 0·1–0·5 % asparagine, and 0·02–0·1 % Tween 80.

Table 3. *Effect of additions to basic medium on growth of* Mycobacterium tuberculosis (*human*)

Glucose % (w/v)	Asparagine % (w/v)	Tween 80 % (v/v)	Growth (mg./ml.) at 10 days
0·05	0·1	0·1	0·2
0·1	0·1	0·1	0·6
0·2	0·1	0·1	0·7
0·5	0·1	0·1	0·7
1·0	0·1	0·1	0·4
0·2	0·02	0·1	0·2
0·2	0·05	0·1	0·6
0·2	**0·1**	0·1	0·8
0·2	0·2	0·1	0·8
0·2	0·5	0·1	0·7
0·2	0·1	0·01	0·2
0·2	0·1	0·02	0·6
0·2	0·1	0·05	0·6
0·2	0·1	**0·1**	0·7
0·2	0·1	0·2	0·3

Figures in black indicate amount selected for final medium.

The original Tween formula contained no added iron salts, but in a modified medium, in which the source of nitrogen is ammonium sulphate, a trace of ferric chloride (0·001 % w/v) was essential to obtain rapid growth of *M. tuberculosis* (Dubos, 1947). It appears that in this medium the Vegex had been omitted. It is possible that in the original Dubos solution the Vegex provided sufficient iron to initiate and maintain growth. It was found, however, that even with the addition of Yeastrel an iron salt greatly improved growth in L.T.S. medium. Table 4 shows the results obtained with decreasing amounts of iron ammonium citrate, and indicates the superiority of this modification in enhancing not only the speed of growth but also the maximum concentration. The amount finally selected was 0·02 mg. iron salts to 1 l. of medium.

Table 4. *Effect of ferrous ammonium citrate on growth of* Mycobacterium tuberculosis (*human*)

Ferrous ammonium citrate (mg./100 ml.)	Growth (mg./ml.) in days		
	4	7	10
0·05	—	0·1	0·3
0·01	tr.	0·5	0·8
0·005	0·3	0·8	1·0
0·002	0·3	0·8	1·1
0·0005	0·2	0·7	0·9
0·0001	0·1	0·4	0·7
None	0·2	0·4	0·7

Figures in black indicate amount selected for final medium.
Symbols as in Table 1.

Table 5. *Growth of* Mycobacterium tuberculosis (*mg./ml.*) *in basic medium plus glycerol*

Glycerol (v/v)	Strain		
	H	B^1	B^2
0·5	1·0	0·8	0·2
1·0	1·1	0·7	tr.
2·0	1·0	0·3	—
4·0	0·2	tr.	—
7·0	tr.	tr.	—
None	0·9	0·6	0·5

H = human; B^1 = bovine from 5 % glycerol medium; B^2 = recently isolated bovine.
Symbols as in Table 1.

Dubos & Davis (1946) noted that the addition of a small amount of glycerol (0·5 %) had no appreciable effect on the growth-supporting properties of Tween medium, but that when the glycerol was increased to 5 % multiplication was poor or completely inhibited according to the purity of the glycerol. The absence of glycerol is of little importance when the tubercle bacillus is being studied, but it might be necessary for the growth of *M. johnei* for which Taylor (1950) has shown that in egg media the optimum concentration of glycerol is 4 %.

In order to examine the influence of glycerol on growth of *M. tuberculosis* in Tween 80 plus L.T.S., various concentrations of a commercial product were tested (Table 5). The growth of the human strain was accelerated by amounts up to 2 %, and a bovine strain, which had been maintained on 5 % glycerol egg medium, by 1 %, but larger amounts had an increasing inhibitory action. A bovine strain recently isolated from milk was much more sensitive to glycerol and was completely inhibited by quantities in excess of 1 %. This suppression of growth may have been due, however, to the direct action of the glycerol on a dysgenic strain and not to the effect of glycerol on Tween 80.

Growth of Mycobacterium johnei *in modified Tween Liquoid-treated bovine serum medium*

Eight strains of *M. johnei* were available for examination. Two were from the A.R.C. Field Station, Compton, one from the Royal Veterinary College, Streatley, three were isolated at Mill Hill during 1939–40, one was from a recent case of Johne's disease in Cheshire, and one was a sheep strain. They were maintained on Dunkin's medium or on a modification of Herrold's egg yolk agar containing *M. phlei.* On the latter medium the rate of growth is slower but the agar base has the advantage that it is firmer than ordinary egg medium and therefore more suitable when weighed amounts of culture are required.

The observations of Davis & Dubos (1948) and of Taylor (1950) that *M. johnei* will not grow in Tween medium were confirmed; in addition, attempts to cultivate the strains in L.T.S. medium, which was most satisfactory for *M. tuberculosis*, were unsuccessful. *M. johnei*, when first isolated, will not grow on egg media or on the surface of liquid media in the absence of growth-promoting factors present in acid-fast bacilli: consequently these results were not unexpected. Davis & Dubos (1948) have reported that small amounts of an extract of *M. phlei* enhance the growth of *M. tuberculosis*, but that larger amounts have an inhibitory effect. *M. johnei* failed to grow in Tween 80 plus albumin V enriched with extract of *M. phlei.*

It was hoped that a glycerol extract of *M. phlei* (Taylor, 1950) would enable *M. johnei* to multiply in L.T.S. medium and such proved to be the case. A 7-day surface growth of *M. phlei* from a 250 ml. flask of 5 % glycerol broth was steamed. The bacterial mass was washed several times, reduced to a fine pulp and taken up in 80 ml. of distilled water and 20 ml. glycerol. The suspension was autoclaved, centrifuged, and the supernatant fluid added to the basal medium before sterilization. Preliminary experiments showed that L.T.S. medium enriched with the phlei extract was satisfactory for the growth of some strains of *M. johnei* when the proportion was not in excess of 4 % or less that 1 % (Table 6). Large amounts of the extract may inhibit growth either by the phlei extract interfering with the protective action of the serum against the breakdown of the Tween 80, as suggested by Dubos, or by the effect of higher concentrations of glycerol in preventing growth.

Multiplication of *M. johnei* in the medium is slow and in primary cultures a visible turbidity may not develop before the 10th to 14th day but, after several subcultures, the rate of growth is accelerated.

Medium sterilized by heating on 3 successive days was less satisfactory than medium sterilized by filtration. Moreover, the improvement in the rate of growth in unheated medium became more marked on subcultivation. In continuous subcultivation, growth was always slower than *M. tuberculosis* and never reached the same maximum intensity (Table 6). There were differences in the ability of the various strains to adapt themselves to the new medium; one strain (R.V.C.), well trained to grow on solid medium, and one recently isolated strain (H) gave a moderate turbidity after 21–29 days in the primary cultures and in the succeeding five passages, but subsequently reached their maximum growth in 12–15 days. One Compton and two Mill Hill strains were more difficult to adapt and were still less active. The other strains, including that from the sheep, have so far failed to show any appreciable growth.

Table 6. *Growth of* Mycobacterium johnei *in basic medium plus glycerol extract of* M. phlei

Glycerol extract % (v/v)	Growth (mg./ml.) at 15th day	
	Strain R.V.C.*	Strain H†
6·0	0·2	tr.
4·0	0·5	0·3
2·0	0·4	0·2
1·0	0·2	tr.
0·5	tr.	tr.
None	—	—

* R.V.C., 10th subculture.
† H, 8th subculture (recently isolated strain).
Symbols as in Table 1.

The fluid usually shows a uniform turbidity but from time to time the growth has become somewhat granular. This change in the character of the dispersed growth seems to be related to certain batches of serum and the cause is unknown. In stained smears the bacilli are not completely dispersed but appear as aggregates varying from 4 to 30 organisms. The bacilli are slightly longer than those from solid medium and may present a beaded appearance.

DISCUSSION

The fundamental studies of Dubos and his co-workers have shown that when a small amount of purified albumin is added to a suitable defined medium containing Tween 80 (a water-soluble ester of oleic acid), *M. tuberculosis* grows rapidly in a dispersed form rather than as a pellicle. In the present investigation it has been demonstrated that the rate of growth of the tubercle bacillus can be accelerated by substituting for fraction V albumin the supernatant portion of serum treated with Liquoid. Dubos & Davis (1946) believe that as Tween 80, in concentrations higher than those recommended for their medium, has a powerful inhibitory action on the multiplication of the tubercle bacillus and as this effect can be overcome by suitable amounts of serum albumin, the latter probably protects micro-organisms against the toxic action of the fatty acids liberated from Tween 80. They suggest that the sparing action of the albumin is the important factor and that there is no evidence of any growth-promoting fraction in the serum. It is possible that the fraction obtained from Liquoid-treated serum has a purely passive effect similar to that of serum albumin. On the other hand, Dubos & Davis have recorded poor growths of acid-fast organisms in Tween medium containing other proteins such as gelatin, serum globulin and casein, which they attribute to the failure of these substances to protect the bacilli against the toxic effects of free oleic acid. If this is the case, one would expect that the sediment from Liquoid-treated serum, which appears to contain nearly all the albumin, would be as effective as fraction V albumin. In fact, it fails entirely to support growth in Tween medium whereas the supernatant fluid, which contains a high proportion of euglobulin and some pseudoglobulin, is fully active. The suitability of the latter fraction might still be ascribed to a sparing action similar to that exerted by albumin, but it would seem equally likely that it contains substances which favour the multiplication of the tubercle bacillus.

A highly purified sample of ferrous ammonium citrate did not improve the rate of growth but was effective in the presence of Yeastrel, whereas a less pure iron salt did not require the addition of Yeastrel. It is possible, therefore, that other elements, such as copper, may be necessary for growth.

The strains of *M. johnei*, which infect cattle, generally grow on a variety of complex egg media containing extracts of acid-fast bacilli. The slow rate of development is a drawback in the study of this group, but as the tubercle bacillus multiplies rapidly in Dubos medium, it was hoped that by adding extracts of *M. phlei*, *M. johnei* would grow in dispersed form. The surprising observation of other workers that such is not the case has been confirmed. The new medium described in this paper is, however, suitable for some strains, irrespective of the period during which they have been grown on conventional media, whereas other strains have so far defied all attempts at adaptation. This observation on resistance to cultivation may be due to strain variations and would fall into line with the findings of Taylor (1945) that some strains are apparently uncultivatable on egg media.

It is clear that the modified medium described is unlikely to be of value in the isolation of Johne's bacillus from natural material, as strains well adapted to artificial cultivation still require several passages in Dubos medium. On two occasions tissues rich in *M. johnei* have been treated with KOH, washed and transferred to the new medium: no evidence of multiplication has been detected.

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A Comparison of a Direct- and a Plate-counting Technique for the Quantitative Estimation of Soil Micro-organisms

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SUMMARY: Estimates of numbers of micro-organisms occurring in three differently manured soils, made by a direct-counting and plating technique, were compared. No correlation was found between the two methods and reasons for the large discrepancy between them are discussed. Contradictory information of the effect of external factors on soil micro-organisms can be given by the two different methods of counting.

Estimates of the total numbers of bacteria in soil obtained by a direct-counting technique devised by Jones & Mollison (1948) are of the order of several thousands of millions/g. soil, while estimates by conventional plating methods are in tens of millions/g., even on the least selective media so far used. The same authors found a difference of the same kind between the quantity of fungal mycelium in soil as estimated by a direct count of number of pieces/g. as compared with colony plate counts, although in this comparison the former count is only 2–6 times greater than the latter.

The effect of external factors such as soil moisture and temperature on the various groups of soil microflora has been investigated by several workers and, while certain correlations have been found, the results have been conflicting. The estimations of numbers of organisms were mainly based on plate counts. The present investigation was undertaken to compare direct counts with plate counts over a period of time, to determine how these varied with soil moisture and temperature and to show whether these two counting methods did, in their different ways, give the same sort of information about the soil population. The soils of three differently manured plots were sampled at intervals (Fig. 1) over the greater part of a year, and determinations made of the moisture content of these samples and of their bacterial, fungal and actinomycetal populations, by plating and a direct-counting technique.

METHODS

Soil sampling. The soil samples were taken from three of the classical plots of Broadbalk field, which has grown wheat continuously for just over 100 years and whose plots have had different manurial treatments over that period. Plot 2 (farmyard manure), plot 3 (no manure) and plot 7 (complete minerals + ammonium sulphate) were sampled at intervals between January and December. Soil was collected by an auger to a depth of 4 in. in 12 areas on each plot; these were bulked and passed through a 3 mm. sieve. From these sieved samples the necessary amounts of soil were taken for the determination of moisture content, for the dilutions required for plating for the estimations of bacteria, fungi and actinomycetes and for preparing films for direct counting. The same samples of soil were used for estimating the numbers of amoebae present; these results were published separately (Singh, 1949).

Media. For plating bacteria and actinomycetes a non-selective medium, based on the 'artificial soil solution' used by Erikson (1947) was prepared; it had the following composition: $Ca(NO_3)_2$, 0·33 g.; $CaSO_4$, 0·8 g.; $MgSO_47H_2O$, 0·7 g.; K_2SO_4, 0·025 g.; K_2HPO_4, 0·005 g.; $NaHCO_3$, 0·2 g.; distilled water to 1 l. To 1 l. of this solution were added: glucose, 0·01 g.; Difco yeast extract, 0·005 g.; $FeCl_3$, trace; water-washed agar, 15 g. The pH was 7·2.

From the sieved soil 10 g. were taken to make the primary suspension from which further dilutions were made in 0·75 % (w/v) NaCl. Five replicate plates were made at a final dilution of 1/250,000 using 15 ml. portions of the above medium per plate. The plates were incubated at 25° for 2 weeks. Although the growth of all organisms was slow on this medium, high counts had been obtained in preliminary tests. A total count of both bacterial and actinomycete colonies was then made with a hand-lens. An actinomycete colony count was made after a microscopic examination of the plates, when finely filamentous colonies, mainly streptomyces and micromonospora, were marked. Filamentous colonies of pasty consistency (*Nocardia* spp.) were not counted.

For plating fungi, Czapek-Dox and Waksman's fungus-agar (Waksman, 1927, p. 19) were used; these media were adjusted to pH 4·6. Later Conn's glycerol sodium asparaginate medium was also used (Fred & Waksman, 1928); it has a lower nutrient value and under test proved to be less selective and to give higher total counts than many other general media for fungi (Brierley, Jewson & Brierley, 1927). Of the sieved soil 25 g. were used to make a primary dilution of 1/10, which was hand-shaken for 20 min. A final dilution of 1/20,000 was used for eight replicate plates having 10 ml. medium/plate. Final counts were made after incubation for 9 days at 25°.

Direct-counting technique. Direct counts of numbers of bacteria and of the number of pieces and total length of mycelium were estimated exactly as described by Jones & Mollison (1948) with one modification. In the original paper the counting of fungal mycelium with a low-power ($\frac{2}{3}$) objective was advocated. In the present investigation counts were made under a $\frac{1}{6}$ objective over the whole field. Although with the lower power a larger number of observations per field is made, giving possibly greater accuracy in the final estimation, there is some difficulty in identifying the smaller fungal fragments and of measuring them. Higher counts are obtained using the $\frac{1}{6}$ objective, as many fine filaments not resolved under a $\frac{2}{3}$ objective are readily seen with the higher power.

Soil suspensions used for the direct and plate counts were made from two portions of the same soil sample but were prepared in different ways. For the direct count the initial suspension was made by grinding a weighed quantity of soil with 15 ml. distilled water, while for the plate count the initial suspension was shaken only. This fundamental difference in technique might be considered a source of discrepancy in the estimate of numbers of organisms, but a small experiment to compare two plate counts, one made by the usual shaken suspension and the other by grinding the soil in the initial suspension before dilution, showed this not to be so. Two soils, a poor soil represented by Broadbalk plot 3, and a rich allotment sample, were used. From each sample 10 g. were used to make a primary suspension, which was shaken, and a final dilution of 1/250,000 was used for plating. A further 2·58 g. from each soil sample was ground up by a glass rod in 15 ml. distilled water, as in the preliminary stages of making soil films (Jones & Mollison, 1948). One ml. was removed to make a final dilution of 1/250,000 for plating, while the remaining 14 ml. were used for the direct-count films. The results of this experiment are shown in Table 1, which shows that though the ground suspension gave the higher plate count in both cases, the difference between the two plate counts was not appreciable and the discrepancy between the plate and total direct count was still 40–50-fold. The difference between the direct colony count and the plate count is approximately 20-fold.

Table 1. *Comparison of direct count with plate count of bacteria using two methods for preparing soil suspensions for the plate count*

	Direct count (millions/g. dry soil)		Plate count (colonies in millions/g. dry soil)			
			Ground suspension		Shaken suspension	
	Aggregates	Total no. bacteria	2 weeks incubation	5 weeks incubation	2 weeks incubation	5 weeks incubation
Allotment soil	2220·1	5145·6	124·4	292·8	112·3	336·9
Broadbalk soil, plot 3	1379·3	2640·8	59·8	122·0	39·5	158·2

The plates from the last experiment were kept, and since they had not dried out were recounted after an incubation period of 5 weeks. Table 1 shows that after 5 weeks the number of colonies had increased to two or three times the number found after the more usual incubation period and that the shaken suspension now gave the higher figures. The discrepancy between the direct and plate counts was here reduced approximately to one of 10–20-fold.

Moisture content. Samples (25 g.) were taken from the sieved soil samples and dried overnight at 105°.

Soil temperature. The temperatures for a depth of 4 in. of soil were taken from those of the Rothamsted meteorological records, which are made on an area about half a mile distant from Broadbalk.

Statistical analysis. Correlation coefficients were used to assess the agreement between the numbers of organisms/g. soil estimated by the two methods and to show whether or not these numbers were dependent on changes in soil moisture and temperature. The correlations and regressions for the three plots were found not to differ significantly amongst themselves and were therefore pooled.

RESULTS

The estimated total numbers of bacteria, fungi and actinomycetes are shown against the sampling dates in Figs. 1 and 2. The numbers of bacteria obtained from plate counts were, with one exception, consistently higher on plot 2 than on plots 3 and 7, which were very similar. By the direct counting method the total numbers of bacteria were 40–50 times higher than by the plating method. Plot 2 again showed consistently higher numbers than plots 3 and 7 which were again similar. It is seen from Table 2 that there is no correlation between the two methods for bacterial counts. A partial correlation calculated after eliminating the effect of soil moisture and temperature was still not significant. This reflects the findings of Taylor (1936), who made a similar comparison between plate counts and a total count obtained by the ratio method of Thornton & Gray (1934).

Table 2. *Correlation coefficients between plate and direct counts of bacteria, fungi and actinomycetes*

	Broadbalk						
	Plot 2	Plot 3	Plot 7	*N*	3 plots aggregated	D.F.	*P*
Correlation coefficients between:							
Direct bacteria and plate bacteria	−0·347	0·032	−0·488	7	−0·286	17	—
Partial correlation—moisture and temperature eliminated	—	—	—	—	−0·331	15	—
Direct bacteria and plate actinomycetes	0·392	0·342	0·622	7	0·433	17	0·05–0·1
Partial correlation—moisture and temperature eliminated	—	—	—	—	0·189	15	—
Plate bacteria and plate actinomycetes	−0·553	0·597	−0·320	7	−0·511	17	0·02–0·05
Partial correlation—moisture and temperature eliminated	—	—	—	—	−0·277	15	—
Direct fungi and plate fungi (Waksman's medium)	**0·725**	0·141	0·015	9	0·324	23	—
Partial correlation—moisture and temperature eliminated	—	—	—	—	0·089	21	—
Direct fungi and plate fungi (Czapek's medium)	0·428	0·622	0·354	8	0·412	19	0·05–0·1
Direct fungi and plate fungi (Conn's medium)	—	—	—	6	0·409	14	—
Direct fungi (no. of pieces) and direct fungi (total length)	**0·805**	**0·731**	**0·815**	11	—	—	0·01

N = number of pairs of observations; D.F. = degrees of freedom; *P* = level of significance.
Significant correlation coefficients in black type.

The highest counts of actinomycetes by the plate method were obtained from the plot treated with farmyard manure while the other two were very similar, with the unmanured plot showing a tendency towards higher numbers (Fig. 1). These figures confirm earlier work by J. Singh (1937), who found that Broadbalk plot 2 carried a much heavier actinomycete flora than plot 3. His figures were much lower than the present ones, probably due in part to his having used a medium of pH 5·4, which is just above the lower limit for actinomycete development, and because in the present investigation one of us (F.A.S.) used microscopic observation for the final identification of each actinomycete colony.

To determine whether bacterial and actinomycete populations fluctuate in a parallel manner, the correlation coefficient was calculated between their respective plate counts and between the bacterial direct count and actinomycete plate count. Direct counts of actinomycetes are not possible at present owing to the difficulty of distinguishing their filaments from fungal hyphae and their spores from bacteria. The aggregated correlation coefficient shows the plate counts to be negatively and significantly correlated, whereas the correlation between the direct bacterial count and the plate actinomycete count is of marginal significance ($P=0{\cdot}1$). However, when the effects of soil moisture and temperature were eliminated, the partial correlations between both pairs of counts showed no correlation (Table 2).

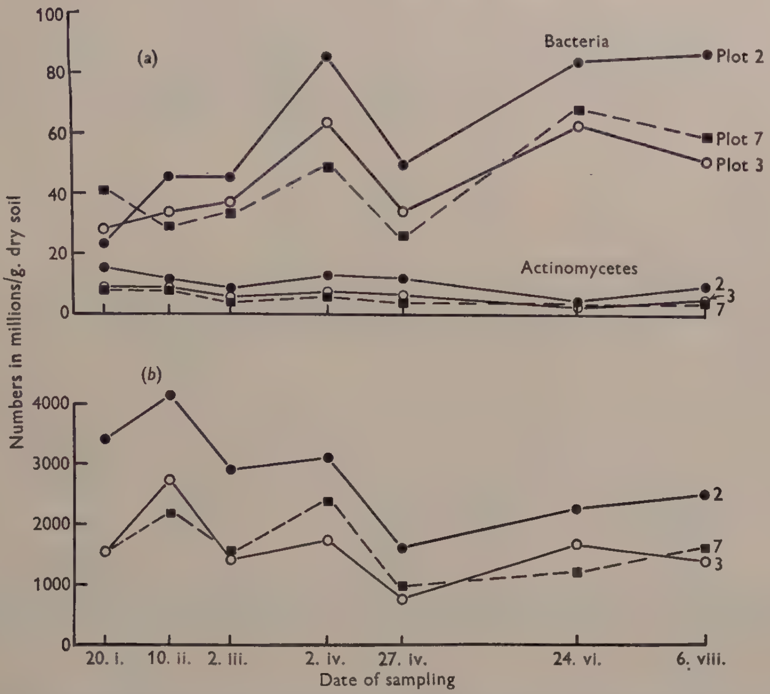

Fig. 1. (*a*) Plate counts of soil bacteria and actinomycetes in the three plots of Broadbalk field on different dates in 1948. (Plot 2, farmyard manure; plot 3, unmanured; plot 7, complete mineral fertilizer.) (*b*) Direct counts of soil bacteria in the same plots as in (*a*).

The numbers of viable fungal particles estimated in thousands/g. obtained by plate counts of colonies on Waksman's medium are shown in Fig. 2. The numbers found in plot 3 were consistently the lowest; plot 7 gave the highest figure on seven of nine occasions. On Czapek's medium the numbers obtained were of the same order; again plot 3 showed consistently the lowest figures and plot 7 the highest on seven of eight occasions. On six of the sampling dates (May–December) fungal counts were also made on Conn's medium. The total numbers fluctuated in a similar manner for all plots but were higher than those obtained on the other three media: plot 2, 280×10^3 to 430×10^3 colonies/g.; plot 3, 130×10^3 to 275×10^3/g.; plot 7, 210×10^3 to 515×10^3/g. Plot 3 gave consistently the lowest count, but plot 7 was not so consistently higher than plot 2.

Direct counts of number of pieces of mycelium/g. of soil are shown in Fig. 2. These estimates are from 2 to 6 times those of the plate counts. Although plot 3 had a tendency to show the lowest figures, this method has shown less consistent differences between the numbers on the three plots. The pieces of mycelium observed and counted were also measured by a micrometer eyepiece and the total length of mycelium was calculated and expressed as m./g. soil. Plot 2 showed 33–65 m., plot 3, 25–57 m., and plot 7, 25–63 m./g. soil.

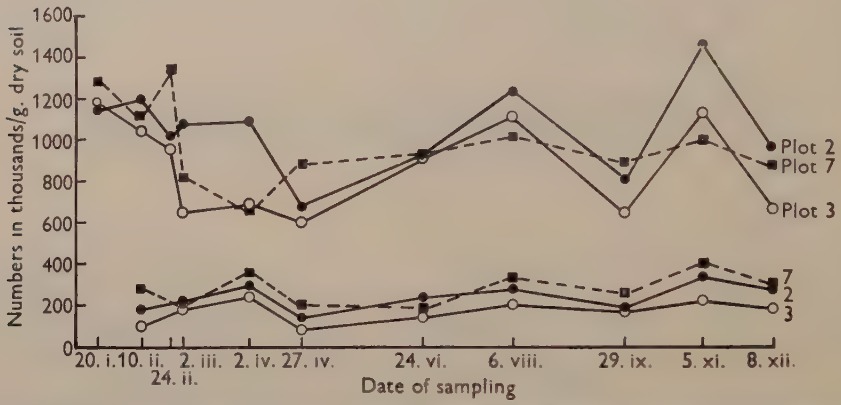

Fig. 2. Upper curves: direct counts of soil fungi expressed as number of pieces of mycelium/g. dry soil. Lower curves: plate counts of soil fungi on Waksman's medium expressed as number of colonies on plate/g. dry soil.

The agreement between the plate and direct methods using the figures for number of pieces of mycelium is not good, as indicated by the correlation coefficients (Table 2); these are positive but only barely significant for plot 2 on Waksman's medium and for plot 3 on Czapek's medium. When the calculation is made on the aggregated observations for three plots, the correlation between the methods is just below the level of significance for all three media. When the effects of soil moisture and temperature are eliminated any relationship between the two counts disappears.

The two estimates of mycelial quantity, number of pieces of mycelium and total length, parallel each other very closely, and a highly significant agreement appears between them for every plot (Table 2). However, the counting of pieces—an easier task than their measurement—does not necessarily give the true picture of the development of mycelium in soil. The average length of pieces measured in one investigation could be much higher than in another, so that while correlation with number of pieces might be good in both, the ratio of number of pieces : total length could be different. This did not reveal itself in the present work, but inconsistency in the grinding of soil for making soil films and differences in fragmentation of hyphal filaments could make this a possibility.

The figures for percentage moisture content and temperature of the soil for the dates of sampling are given in Table 3. In investigating the effect of soil moisture and temperature on the numbers of bacteria and of fungi, it was found that direct and plate counts often indicated conflicting relations. For instance, a positive significant correlation was found between soil temperature and bacterial plate counts, whereas a negative significant one obtained between soil temperature and bacterial direct counts. If the interaction of moisture be taken into account, no correlation is then found between bacterial direct count and temperature (Table 4). The effect of moisture on bacterial numbers assessed by plate counting is not significant but tends to be an inverse relation, while by direct counting it is a positive and highly significant effect, when judged by a total correlation. With the elimination of the effect of temperature, both methods of counting show a positive and barely significant relation with moisture. Further data on the effect of moisture and temperature on fungi and actinomycetes are given in Table 5. Actinomycetes show a small, positive total correlation with moisture, significant in plot 7, and a high negative

Table 3. *Soil moisture and temperature*

	Moisture content (%)			
Date	Plot 2	Plot 3	Plot 7	Soil temperature
20. i. 48	23·45	18·99	20·59	0·7°
10. ii. 48	19·88	15·72	17·08	5·5°
24. ii. 48	20·70	16·90	17·50	0·6°
2. iii. 48	18·44	13·16	14·24	3·3°
2. iv. 48	20·50	15·12	16·92	5·1°
27. iv. 48	9·76	8·36	8·68	11·3°
23. vi. 48	17·36	14·40	15·48	13·5°
6. viii. 48	15·60	12·20	13·40	17·7°
29. ix. 48	16·28	11·96	12·60	13·5°
5. xi. 48	21·00	16·00	16·80	6·8°
8. xii. 48	22·30	16·80	19·00	7·8°

Table 4. *Correlation coefficients between plate and direct counts of bacteria and soil moisture content and temperature*

	Broadbalk						
	Plot 2	Plot 3	Plot 7	*N*	3 plots aggregated	D.F.	*P*
Correlation coefficients between:							
Direct bacteria and moisture content	0·618	0·480	0·444	10	**0·533**	26	0·01
Partial correlation—temperature eliminated	—	—	—	—	0·327	25	0·05–0·1
Plate bacteria and moisture content	−0·309	−0·065	0·185	7	−0·118	17	—
Partial correlation—temperature eliminated	—	—	—	—	0·406	16	0·05–0·1
Direct bacteria and temperature	**−0·655**	−0·347	−0·448	10	**−0·496**	26	0·01
Partial correlation—moisture eliminated	—	—	—	—	−0·245	25	—
Plate bacteria and temperature	0·712	0·480	0·569	7	0·591	17	0·01
Partial correlation—moisture eliminated	—	—	—	—	0·670	16	0·01

N=number of pairs of observations; D.F.=degrees of freedom; *P*=level of significance.
Significant correlation coefficients in black type.

correlation with temperature, significant in plot 7 and for the three plots together. The direct fungal count also shows a significant correlation with moisture but not for temperature. The plate counts of fungi on Czapek and on Waksman media give total correlations with moisture and temperature which are not very similar, although the figures for the individual plots for each medium are fairly consistent, with the notable exception of plot 7 on Czapek medium. A much greater number of observations would have to be made before any explanation of these results could be offered.

Table 5. *Correlation coefficients between fungi and actinomycetes and soil moisture content and temperature*

	Broadbalk						
	Plot 2	Plot 3	Plot 7	*N*	3 plots aggregated	D.F.	*P*
Correlation coefficients between:							
Plate actinomycetes and moisture content	0·278	0·429	**0·800**	7	0·448	17	0·05–0·1
Plate actinomycetes and temperature	−0·586	0·687	**−0·712**	7	**−0·633**	17	0·01
Direct fungi and moisture	0·590	0·544	0·418	11	**0·517**	29	0·01
Direct fungi and temperature	−0·268	−0·139	−0·404	11	−0·266	29	—
Plate fungi (Waksman's medium) and moisture	**0·678**	0·424	0·518	9	**0·550**	23	0·01
Plate fungi (Czapek's medium) and moisture	0·273	0·695	−0·005	8	0·142	19	—
Plate fungi (Waksman's medium) and temperature	−0·127	−0·125	−0·161	9	—	—	—
Plate fungi (Czapek's medium) and temperature	0·356	0·561	0·587	8	**0·453**	19	0·02–0·05

N = number of pairs of observations; D.F. = degrees of freedom; *P* = level of significance.
Significant correlation coefficients in black type.

DISCUSSION

In the present investigation direct counts of numbers of bacteria and fungi were always much higher than those of corresponding plate counts, and the two methods gave different information about their fluctuations. Several factors may contribute to the large discrepancy in numbers given by the two methods: (1) clumps of bacteria which remain aggregated in spite of the shaking that precedes plating; (2) staining of non-viable particles in direct count preparations; (3) failure to distinguish between bacteria and actinomycete spores, and between fungal and actinomycete filaments in stained films; (4) competition on plates; (5) selectivity of plating media and non-cultivation of obligate anaerobes.

The frequency with which plate colonies develop from aggregates of bacteria rather than from individual cells is not easy to assess but there is strong evidence that this must occur. It is shown in Table 6 that the number of observations of cells in 'colonies' of two or more was about the same as that of single cells. These small aggregates had resisted grinding in the preparation of the soil films, and it is unlikely that similar ones would have disintegrated into their component cells during the shaking that preceded plating. If, as is probable, aggregates such as those observed in soil films gave rise to only single colonies on plates, this alone would have made the direct count approximately double the plate count. Further, a minute soil particle bearing several individuals or even aggregates of different species might give only one colony on a plate. The occurrence of impure colonies on plates suggests strongly that this may frequently happen.

Table 6. *Analysis of bacterial aggregates occurring on soil films*

	No. aggregates per slide							
	Broadbalk (plot 3)				Allotment			
Slide no.:	1	2	3	4	1	2	3	4
No. of cells in aggregate								
1	62	49	61	63	85	82	82	89
2	35	30	31	16	31	32	34	48
3	4	6	3	6	7	6	8	5
4	9	12	2	3	9	15	11	15
5	4	2	3	2	2	7	9	3
6	2	3	1	2	6	4	5	5
7	1	–	–	2	–	–	1	1
8	–	2	2	–	1	1	1	4
9	–	–	–	1	2	–	–	–
10	–	–	1	–	1	–	–	1
11	–	–	–	1	–	–	–	–
12	–	–	–	–	–	–	2	1
14	–	–	–	–	–	–	2	1
16	–	–	–	–	–	1	–	–
17	–	–	–	–	–	–	–	1
20	–	–	–	–	–	1	–	–
21	–	–	–	–	–	1	–	–
53	–	–	–	–	1	–	–	–
Total no. of aggregates	117	104	104	96	145	150	155	174
Total no. of cells	219	219	187	181	339	348	360	397
Ratio: $\frac{\text{Aggregates}}{\text{Total cells}}$	0·53	0·47	0·56	0·53	0·43	0·43	0·43	0·44

The staining of dead cells in direct count preparations may be expected to introduce a serious error. That many hyphal pieces observed under the microscope seem empty of protoplasmic contents and are purplish in colour instead of the intense blue shown by known actively growing mycelium was pointed out by Jones & Mollison (1948). To a lesser degree bacterial cells of varying intensity of blue can be seen, but to attempt to separate viable from non-viable particles by depth of colour would be too arbitrary a procedure. It is interesting to note that Taylor (1936), in experiments with soil cultures of bacteria (all of which were able to grow on a soil extract agar), found a plate count on this same medium to be 36–61 % of a direct count estimated by the ratio technique of Thornton & Gray (1934), which gives counts of a magnitude similar to that given by the method used in the present work. As shown in Table 6 a difference of 36–61 % can be accounted for if clumps of several cells develop into single colonies on plates, and thus cannot necessarily be attributed to the staining of non-viable cells. On the other hand, the number of non-viable but stainable cells may accumulate with time and may therefore be greater in a field soil than in an artificial soil population incubated for the relatively short periods used by Taylor. At present, the separation of viable from non-viable organisms in direct counting remains impracticable.

The difficulty of distinguishing bacteria from actinomycete spores is well known. These spores abound in soils, and there is strong evidence that in some soils actinomycetes may be present largely in this form (Skinner, 1951). Allowing that some actinomycetes may not develop on plates, it seems possible that almost the whole actinomycete count of 10–15 million/g. soil is included in the direct bacterial count. However, unless there are vast numbers of dead actinomycete spores present, this source of error cannot be a large one as the actinomycete plate count is small compared with the direct bacterial count.

Competition between colonies on plates and the antagonistic suppression of one species by another are factors which may cause a lower plate count, but no evidence can be presented to show that they were operating in the present investigation.

It has been shown that the growth of single colonies from bacterial aggregates would account for a plate count only half the corresponding direct count. However, this alone is inadequate to explain the enormous discrepancy between numbers given by the two methods. The most probable further causes of this discrepancy would seem to be the counting of non-viable particles in soil films (tending to increase the direct count) and selectivity of the plating medium, a well-known limitation of all plating techniques tending to reduce the plate count.

The ratio fungal plate count : direct count is only 1 : 2–6; selectivity of the plating medium and the direct counting of non-viable fragments could explain this small difference satisfactorily. In view of Skinner's (1951) results, it seems unlikely that actinomycete fragments contribute appreciably to the fungal direct count. It is important to remember that different soil conditions may encourage sporulation of fungi rather than mycelial growth. For this reason a plate count may give a false picture of mycelial development while a direct count may give a truer analysis.

Many studies on the effect of external factors on the fluctuating numbers of micro-organisms have been made, and although the results have often been contradictory, most workers consider that variations in soil moisture content must have some effect on the micro-population, although Taylor (1936) showed that fluctuations of bacterial numbers took place while soil moisture content and temperature were kept constant. It is evident that neither direct nor plate counting records accurately changes in the total numbers of soil micro-organisms. In direct counts such fluctuations may be masked by an accumulation of non-viable but stainable cells. The selectivity of plating media and the fact that here plate counts of bacteria were positively and direct counts negatively correlated with temperature suggest that the plate count is an estimate of one element of the soil population, which reacts to external factors differently from the total population. With these considerations in mind it is easier to understand the absence of correlations between plate and direct counts.

Plate counting is a well-established method, perhaps of greatest value for qualitative investigation. We consider that the new direct-counting technique, though it undoubtedly gives an overestimate of the viable population, has proved reliable for quantitative investigations. It is rapid, and as both bacteria and fungi can be examined in the same soil film, it saves labour and equipment necessary for plating experiments.

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Selection of Penicillin-sensitive Mutants of *Escherichia coli* following Ultraviolet Irradiation

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SUMMARY: By taking advantage of the long lag period before minimal therapeutic concentrations of penicillin kill bacteria, five mutants of the K12 strain of *Escherichia coli* (*Bacterium coli*) have been isolated following ultraviolet irradiation, which were 10–50 times more sensitive to penicillin than K12. This supports the theory that development of antibiotic-resistant forms is by mutation and selection.

It appears from the work of Demerec (1945, 1948) that in any given population of penicillin-sensitive organisms there are a few more resistant ones and these are believed to arise by spontaneous mutation. There would seem to be no strong reason why the same population of bacteria should not contain a few organisms more sensitive than the rest; the main difficulty in demonstrating this is one of selecting a minute proportion of sensitive ones from a mass of more resistant bacteria.

Many people have shown that strains of bacteria with an induced resistance to penicillin will sometimes become more sensitive simply by repeated subculture in the absence of the drug (Todd, Turner & Drew, 1945; Chain & Duthie, 1945). Very little work has been done on these lines with organisms which are naturally resistant to penicillin, even before contact with the drug.

One case where a naturally resistant organism has become somewhat more sensitive is that reported by Plough & Grimm (1949); in the course of testing auxotrophic mutants derived from *Salmonella typhimurium*, by ultraviolet irradiation they isolated a cysteine-requiring mutant which was 4–16 times more sensitive to penicillin than the original, depending on the medium used for testing. Recently Voureka (1951) has reported the production of variants from *Escherichia coli* (*Bacterium coli*) by growth in presence of antiserum and chloramphenicol; one of these, variant β, was almost 10 times more sensitive to penicillin than the original.

A consideration of the early compiled tables of penicillin sensitivities of various organisms is striking for the very wide variations in sensitivity within many species. Thus, strains of *Esch. coli* vary in sensitivity from 15 to 5000 units per ml. (u./ml.) (Florey *et al.* 1949). It seems likely that these differences have not been induced by previous contact with the drug (as is the case with most resistant staphylococci), at any rate, these strains do not change in penicillin sensitivity merely on repeated subculture in drug-free media. It seems quite probable that these different strains have arisen in nature by mutations, and perhaps similar changes can be brought about in the laboratory by methods designed to increase the number of mutants, e.g., irradiation with ultraviolet light.

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The peculiar bacteriostatic and bactericidal effects of penicillin are such that selection of any more sensitive forms appeared possible. Parker & Marsh (1946) have shown that the viability of staphylococci after exposure to penicillin begins to decrease only after a lag period, which is longer for lower penicillin concentrations; if the penicillin is removed before the end of this lag period the organisms will begin to grow after a few hours' delay. This suggested the possibility of selecting sensitive variants by plating out on a medium containing as high a concentration of penicillin as will allow the wild type to grow freely; if the penicillin is then removed by addition of penicillinase, any more sensitive variants could begin to grow provided that the concentration of the drug to which they had been exposed was near to the minimum effective dose for those variants, and that the time of exposure had not been too long. By this means the wild type would have a start of several hours over any sensitive variants which would then be likely to appear as very small colonies.

The K12 strain of *Esch. coli* was chosen for the present work for the following reasons:

(*a*) Most of the work on bacterial genetics has been done with K12 and it has been shown to recombine (Lederberg, 1947).

(*b*) It will grow readily on inorganic salts plus glucose.

(*c*) Because of its minimal growth requirements there is the possibility that any metabolic differences in sensitive organisms would be more readily detected (Davis, 1950).

EXPERIMENTAL

The K12 strain of *Esch. coli* was used; this will grow on 60 u. penicillin/ml., but not on 90 u./ml. The size of inoculum has very little effect on the apparent sensitivity so that active penicillinase production can be ruled out. Cultures were grown on nutrient broth overnight; 3 ml. of such a culture were irradiated for 1 min. in an open Petri dish by ultraviolet light (this leaves approx. 10^{-4} of the organisms viable); dilutions of the irradiated culture were made in broth; usually 1/50 and 1/500. These dilutions were then incubated for 1 hr. to allow for delayed phenotypic expression (Davis, 1950), then, 0·2 ml. of each dilution was spread on to each of ten 6 in. nutrient agar plates containing 500 u. penicillin/ml. After 8 hr. the plates were removed from the incubator and sprayed with a solution containing 1000 u. penicillinase (Schenley Labs. Inc.)/ml.; after another 4 hr. or so, contact photographic prints were made of all the plates; by this time the K12 colonies had grown to a reasonable size. Incubation was then continued for a further 24 hr. making a total period of incubation of about 40 hr. At this time the plates were examined for secondary colonies by placing them in position on the photographic print and shining blue light through them from beneath; a yellow light shining from above enabled one to pick off any secondary colonies, which appeared yellowish and were usually minute, in contrast to the others which were bluish. Usually plates with a total of 400–500 colonies yielded one or two secondary colonies. These secondary colonies were picked off, inoculated into small broth tubes and incubated for 6–8 hr. at which time they were streaked out on penicillin ditch plates containing 300 u. penicillin/ml. of agar in the ditch; a streak of the original K12 (8 hr. culture) was included for comparison.

Penicillin sensitivities of the isolated variants were further tested by tube assay in nutrient broth using bulk dilutions of penicillin of 50, 25, 12, 6, 3 and 1 u./ml., in a total of 2 ml. The tubes were read after 16 hr. Inocula of approximately 1000 organisms were used; with much larger inocula all strains appeared to have sensitivities approaching that of K12, presumably due to the early appearance of reverse mutants.

Minimal medium was that of Davies & Mangioli (1950).

RESULTS

From the first 140 secondary colonies only one (A) was significantly more sensitive than K12. This was then used as the stock strain for further irradiation instead of K12, the penicillin content of the plates being decreased to 10 u./ml., to allow normal growth of this organism. From 190 secondary colonies using A as stock, four more sensitive variants were isolated (2A, 3A, 4A and 5A). The strain 4A was then used for stock and the penicillin concentration of the plates decreased to 2 u./ml. No more sensitive strains appeared from 105 secondary colonies, so the penicillin concentration was decreased to 0·5 u./ml., again without success; finally a concentration of 0·1 u./ml. was tried without producing any more sensitive strains than 4A. Altogether, 200 secondary colonies from 4A were tested.

Table 1. *Titration of variants in nutrient broth with inoculum of c.* 1000 *organisms/tube*

		Penicillin concentrations in units/ml.					
Organism	50	25	12	6	3	1	0
K12	++	+++	+++	+++	+++	+++	+++
K12	+++	+++	+++	+++	+++	+++	+++
A	–	–	+	+++	+++	+++	+++
A	–	–	–	++	+++	+++	+++
2A	–	–	–	+	+++	+++	+++
2A	–	–	–	+	+++	+++	+++
3A	–	–	–	+	+++	+++	+++
3A	–	–	–	+	+++	+++	+++
4A	–	–	–	–	+++	+++	+++
4A	–	–	–	–	+	+++	+++
5A	–	–	–	–	–	+	+++
5A	–	–	–	–	–	++	+++

+ + + = good growth to – = no growth.

Table 1 shows the sensitivity tests for these five variants by tube assay with minimal inocula.

The variants were tested against coli phages T1, T2, T3 and T4; like K12 they were all sensitive to T1, T2 and T4 and were not affected by T3.

All the variants have been subcultured at least 10 times and 4A has been subcultured over 20 times in broth without loss of sensitivity; at the end of the 20 subcultures 4A showed a fair proportion of more resistant colonies and had to be purified.

As a control to the experiments a K12 culture was diluted and plated out on penicillin agar without ultraviolet irradiation; secondary colonies did appear in about the same numbers as with the irradiated strain, but out of 150 secondary colonies tested none had any greater sensitivity than K12.

DISCUSSION

The great majority of the secondary colonies had the same sensitivity as the parent stock, and one must assume that they were derived from organisms which were in an unfavourable state for growth and required a long lag before commencing active growth. They may be analogous to the persisters of Bigger (1944). However, the minority of the secondary colonies were more sensitive variants and the numbers were roughly one from 2×10^4 surviving bacteria (or possibly 2×10^8 original bacteria). It will be recalled that Demerec (1945) arrived at a figure of 1 in 10^7 for spontaneous penicillin-resistant mutants in staphylococcus. Insufficient numbers of bacteria were examined to form a really good non-irradiated control, so that there is no clear proof that these sensitive strains are produced as a result of the ultraviolet irradiation. Nevertheless, in so far as it is extremely unlikely that any adaptation will go against the pressure selection gradient, these more sensitive strains would seem to be true mutants of K12.

It is interesting that in spite of the sensitivity having been increased by 10–15 times from K12, all of the mutants except 5A could grow on minimal medium. In the case of penicillin there is a general tendency for resistant bacteria to be more nutritionally independent than sensitive ones. Gale & Rodwell (1949) have trained sensitive staphylococci to grow with fewer amino-acids in the medium and in the course of this training the organisms became concurrently penicillin resistant, in spite of having had no contact with the drug. This seems analogous to the experiments of McIlwain (1943) in training *Corynebacterium diphtheriae* to grow without pantothenic acid when it became concurrently insensitive to pantoyltaurine, and one might argue by analogy that penicillin antagonizes or in some way interferes with the absorption of one or more amino-acids (Gale & Rodwell, 1949). It has, however, been pointed out that there are bacteria, notably *Bacillus subtilis*, which are extremely sensitive to penicillin even though they grow on completely inorganic media with glucose (Hunter & Baker, 1949).

During the course of this work I had the opportunity to test 161 temperature-sensitive primary mutants of K12 for penicillin sensitivity; all of the mutants would grow on minimal medium at 25° but required added nutrilites for growth at 37°. Eight of these primary mutants were more penicillin sensitive than K12; Table 2 shows the growth requirements of these organisms (Leupold, U., personal communication).

Since these are primary mutants the increased sensitivity must be associated with the increased growth needs; it is, however, clear that there is no common requirement for all these mutants.

Table 2. *Growth requirements and penicillin sensitivities of K12 mutants*

No.	Requirements at 37°	Penicillin sensitivity (μ./ml.)
2520	Methionine + lysine	3
2463	Methionine or valine or α-aminobutyric acid	1
580	Lysine or isoleucine + valine or α-aminobutyric acid	3
1253	Threonine or yeast nucleic acid	6
1424	Methionine	6
2430	Unknown	3
998	Unknown	3
1779	Unknown	3

We have in all, then, thirteen mutants of *Esch. coli,* all considerably more sensitive to penicillin than K12; nine of these mutants associate their sensitivity with increased nutritional requirements of various sorts, four do not. Hunter & Baker (1949) have shown that Gale's original hypothesis that 'The sensitivity of a cell to penicillin is determined by the degree to which its growth processes are dependent upon assimilation of preformed amino-acids rather than their synthesis', is no longer tenable when applied to different bacterial species. The present results show that even within one species the hypothesis does not always hold true.

It is, nevertheless, difficult to avoid the belief that there is a relationship between penicillin sensitivity and amino-acid assimilation or metabolism; any derangement of amino-acid metabolism in the mutants A, 2A, 3A and 4A, however, must be an internal one as it is not reflected externally in the form of increased nutritional requirements.

It appears that there are many different alterations in bacterial metabolism which can affect penicillin sensitivity, and since many of these (Davis, 1950) enzymic reactions of metabolism have been shown to be genetically controlled, it is clear that, indirectly at least, variations in the degree of penicillin sensitivity are under the same genetic control.

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Interchangeability of Quinolinic and Nicotinic Acids as Growth Factors for a Pseudomonad Oxidizing Nicotinic Acid

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SUMMARY: A pseudomonad obtained from a nicotinic acid enrichment culture had a growth-factor requirement satisfied by high concentrations of nicotinic acid or by minute amounts of quinolinic acid, 3-hydroxyanthranilic acid, or nicotinamide. Nicotinic acid, kynurenin sulphate, and probably 3-hydroxyanthranilic acid, can serve as sole carbon sources. The oxidation of nicotinic acid is adaptive. The finding that low concentrations of nicotinic acid fail to satisfy the growth-factor requirement is discussed.

The paucity of records of auxotrophy in pseudomonads may reflect the selection exercised by isolation media devoid of growth factors. Pseudomonads requiring aneurin were isolated from enrichments with uracil and thymine. The utilization as carbon sources of metabolites of the anthranilic acid-tryptophan-nicotinic acid series may prove of taxonomic value in *Pseudomonas*.

Studies of the oxidation of tryptophan (Stanier & Tsuchida, 1949) and of other aromatic compounds (Stanier, 1950; Happold, 1950) by bacteria suggest that dissimilation of these compounds may proceed via pathways (in reverse) common to biosynthesis. Such applications of micro-organisms as tools for investigating intermediary metabolism prompted the present investigation of isolation methods. This paper includes a discussion of enrichment techniques and a report of the investigation of oxidative and biosynthetic pathways in a pseudomonad isolated by a modification of the usual enrichment culture technique.

The usual method of isolation is to enrich an inorganic medium with the substrate of interest, inoculate with soil, and eventually obtain the desired organism from the enrichment culture. This procedure is limited in that it selects against organisms which are auxotrophic (i.e. growth-factor-requiring). The importance of auxotrophy is underscored by the experience of den Dooren de Jong (1926) who failed to obtain hexanol-oxidizing bacteria by the simple enrichment technique. Foster (1944) later showed that many photosynthetic pseudomonads (cf. van Niel, 1944) utilize hexanol, and they require growth factors (Hutner, 1950). *Euglena gracilis*, an algal flagellate which also can utilize hexanol and grow heterotrophically in darkness, requires aneurin and vitamin B_{12} (Hutner & Provasoli, 1951).

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Compounds involved in the biosynthesis of nicotinic acid, i.e. anthranilic acid, tryptophan and nicotinic acid, served as test substrates in our studies. The biosynthesis of these compounds is under scrutiny in *Neurospora* and in animals, and material for comparison is thus available. Furthermore, as Koser & Baird (1944) had found that nicotinic acid was an excellent enrichment substrate for pseudomonads, it became of interest to determine whether the provision of growth factors in minimal amounts would permit the isolation of auxotrophic pseudomonads. The structures of the various compounds related to nicotinic acid which are referred to in this paper are shown in Fig. 1 (p. 280).

One of our enrichment cultures yielded a pseudomonad having a growth-factor requirement satisfied by high concentrations of nicotinic acid (pyridine-3-carboxylic acid) or by quinolinic acid (pyridine-2, 3-dicarboxylic acid) in minute concentrations. Since this bacterium has been applied as an assay organism for quinolinic acid (Jakoby & Bonner, 1951), and since quinolinic acid has been reported to be a possible intermediate in the biosynthesis of nicotinic acid, the physiology of the organism merits special description.

METHODS

Unless otherwise stated, the cultural methods were those previously described (Hutner, 1950).

Isolation procedure. The isolation steps were the following: (*a*) inoculation of the enrichment medium with soil or mud; (*b*) inoculation of a flask of enrichment medium with a loopful of culture from *a*; (*c*) streaking growth from *b* on to enrichment agar; (*d*) picking colonies on to agar slopes of enrichment medium; and (*e*) selection of cultures oxidizing the substrate in liquid media. Flagella staining by the silver method of Novel (1939) proved simple and reliable for identifying polar flagellate rods.

Culture media. The basal medium (Table 1) finally adopted both for enrichment cultures and for pure culture studies permitted good growth when supplemented with appropriate substrates. The substrate employed in growth-factor experiments was sodium succinate.$6H_2O$ (0·2 %) and NaH glutamate (0·125 %). Ethylenediamine tetra-acetic acid (EDTA) was used as a metal carrier to insure the availability of essential but precipitable metals (Hutner, Provasoli, Schatz & Haskins, 1950). The media for enrichment cultures were supplemented with growth factors at the following concentrations (mg./100 ml. final volume): adenine, 1·0; uracil, 1·0; DL-methionine, 1·0; aneurin, 0·05; Ca pantothenate, 0·05; nicotinic acid, 0·05; pyridoxine, 0·02; riboflavin, 0·02; folic acid, 0·01; biotin, 0·002.

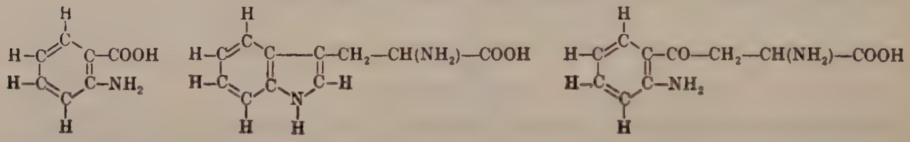
Basal media and stock solutions of nutrients were preserved with a mixture consisting of (v/v) 1 part chlorobenzene, 2 parts *n*-butyl chloride, and 1 part 1 : 2-dichloroethane; autoclaving removed the preservative. No contaminations in stock solutions were encountered while using it over a 2-year period.

Table 1. *Basal medium for growth-factor studies*

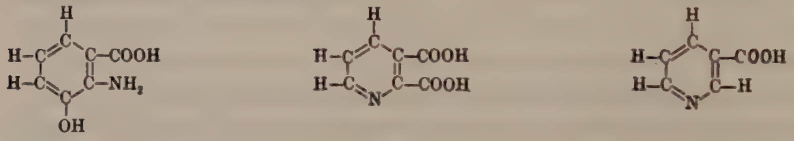
	g.
K_2HPO_4	0·02
Ethylenediamine tetra-acetic acid (EDTA)	0·05
$MgSO_4.7H_2O$	0·08
NH_4Cl (omitted in enrichment media)	0·02
Metals (mg.): Ca, 2·0; Zn, 5·0; B, 2·0; Mn, 1·4; Fe, 1·0; Mo, 0·6; Cu, 0·4; Co, 0·4.	

Distilled water to 100 ml.; pH adjusted to 6·6–6·8 with KOH. The concentration of metals refers to the metal content of the salts used. Calcium was dispensed from a solution made by dissolving $CaCO_3$ in a minimal quantity of HCl. Free EDTA is sparingly soluble but readily dissolves as the medium is neutralized.

L-Kynurenin sulphate and 3-hydroxyanthranilic acid were sterilized by filtration. An Eastmen preparation of quinolinic acid was re-crystallized from 40 % acetic acid until its nicotinic acid content as measured with *Lactobacillus arabinosus* was insignificant.



Anthranilic acid Tryptophan Kynurenin



3-Hydroxyanthranilic acid Quinolinic acid Nicotinic acid

Fig. 1. Structural formulae of compounds used.

Culture conditions. Enrichment media were distributed in shallow layers in 125 ml. flasks and incubated at 22–30°. Growth experiments with the 'quinolinic' pseudomonad were conducted in 50 ml. flasks containing 10 ml. of medium and incubated at 30°. As was anticipated from the oxidative character of the organism, growth was greater and more rapid in shaken cultures. Stock cultures were stored at 6° on agar slopes covered with paraffin oil. The 'quinolinic' pseudomonad is in the American Type Culture Collection (No. 108,38).

Manometric technique. O_2 and CO_2 were measured by the direct method of Warburg. Cells for these experiments were incubated on a shaker for 2 days at 30°. The cells were collected by centrifugation, washed twice in M/60 phosphate (pH 7·0), and suspended in the same buffer.

RESULTS

The 'quinolinic' pseudomonad

Growth-factor requirement. The 'quinolinic' pseudomonad was obtained from a culture enriched with 0·2 % nicotinic acid. Its auxotrophy was detected when the succinate glutamate medium failed to support growth. Growth was obtained when this medium was supplemented with relatively large quantities of nicotinic acid. Quinolinic acid and 3-hydroxyanthranilic acid were efficacious in extremely small amounts (Fig. 2). L-Tryptophan, kynurenin sulphate and anthranilic acid had very slight activity; indole was inhibitory.

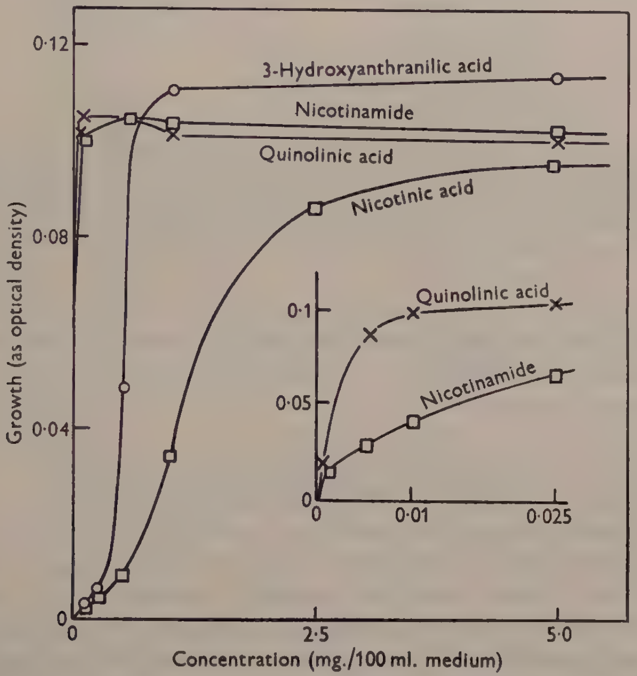


Fig. 2. Utilization of compounds of the anthranilic acid-tryptophan-nicotinic acid series as growth factors in succinate glutamate medium. The insert graph (lower right-hand corner) is an expansion of the quinolinic acid and nicotinamide curves in the large graph.

Carbon sources. When the organism was grown on the usual succinate glutamate medium with quinolinic acid as the 'vitamin source', the organisms were not adapted to the oxidation of nicotinic acid or other intermediates in its synthesis. Adaptation to oxidize nicotinic acid required approximately 2 hr. Bacteria grown on nicotinic acid as both carbon and vitamin source were adapted to the oxidation of nicotinic acid alone. Although 3-hydroxyanthranilic acid was not tested as a carbon source (because of its scarcity) the increase in oxygen uptake in its presence (Table 2) indicated that it probably can function as such.

Nicotinic acid at 10 mg./100 ml. allowed visible growth. L-Tryptophan, kynurenin sulphate and anthranilic acid allowed slow growth at higher concentrations. Nicotinamide and quinolinic acid would not serve as carbon sources.

The theoretical R.Q. for complete oxidation of nicotinic acid is 1·1. The R.Q. actually observed for concentrations of nicotinic acid varying from 0·1 to 3 μM. was 0·65–0·66. In the presence of 2 : 4-dinitrophenol, a reagent considered to uncouple oxidation from assimilation, the R.Q. was increased to 0·87, with a concomitant increase in oxygen consumption from 3·6 μmole O_2 to 5·1 μmole O_2 per μmole nicotinic acid. That a portion of the nicotinic acid is assimilated is further shown by the ability of nicotinic acid to serve as sole source of fixed carbon in growth experiments.

Table 2. *Manometric measurements of the oxidation of substrates related to quinolinic acid*

Substrate	μmole O_2/μmole carbon source
Quinolinic acid	0
Nicotinamide	0·2
3-Hydroxyanthranilic acid	2·6
Kynurenin.SO_4	3·6
Nicotinic acid	3·6

Final values were taken when the oxidation rate was equal to that of the endogenous respiration. Small amounts of nicotinic acid in the range tested (0·1 μM. to 3 μM.) were oxidized to the same extent as larger amounts. The bacteria for this experiment were grown with quinolinic acid as vitamin source and succinate + glutamate as substrate.

Pseudomonads from enrichment cultures containing tryptophan, anthranilic acid, nicotinic acid, or other carbon sources

The anomalous ability of the 'quinolinic' pseudomonad to oxidize vigorously some of the compounds which serve it as growth factors had no counterpart in any of twenty-nine other pseudomonads obtained from nicotinic acid enrichment cultures. Its auxotrophy is unique: none of twenty-one isolates from anthranilic acid (0·1 %) enrichments, nor any of another twenty-one isolates from DL-tryptophan (0·2 %) enrichments proved to be auxotrophic. This raised the question whether the present isolation technique is adequate for obtaining other specialized oxidative organisms. Den Dooren de Jong's (1947) studies with simple aliphatic amines, particularly diethylamine, provided a test case. No difficulty was experienced in the present experiments in obtaining these organisms with either 0·5 % diethylamine.HCl or 0·4 % ethylurea. On test, two strains each of *Protaminobacter rubrum* and *P. alboflavum* thus isolated in the presence of growth-factor supplement were not auxotrophic. These enrichments yielded, however, a culture which gave rise to distinct red and white colonies which could not be grown apart from each other. Dr W. Vishniac has informed us that he has experienced a similar difficulty with diethylamine enrichment cultures.

Some of the pseudomonads isolated from cultures enriched with uracil (0·2 %) exhibited a requirement for aneurin.

DISCUSSION

Auxotrophy in pseudomonads

The isolation of the 'quinolinic' pseudomonad and aneurin-requiring pyrimidine oxidizers shows that auxotrophy in pseudomonads is not confined to photosynthetic forms. These experiments also indicate that an enrichment culture technique which provides growth factors, favours the isolation of a more varied representation of the organisms present in nature. Dr J. W. Foster has informed us of further examples of auxotrophy in this group: biotin is required for the growth of a pseudomonad capable of oxidizing lumichrome, and another which can oxidize riboflavin. West & Lochhead (1940) showed that many bacteria in the rhizosphere are auxotrophic; auxotrophic pseudomonads, it appears, may constitute a significant portion of the microflora in the rhizosphere.

None of the twenty-nine other cultures obtained by nicotinic acid enrichment was auxotrophic. The utilization as carbon sources of compounds in the anthranilic acid-tryptophan-nicotinic acid series may reflect valid taxonomic distinctions. Of the other twenty-nine nicotinic acid oxidizers, five oxidize tryptophan but not anthranilic acid, eight oxidize both tryptophan and nicotinic acid, three oxidize anthranilic acid but not tryptophan, and thirteen neither tryptophan nor anthranilic acid. Similarly, tryptophan-utilizing organisms and the anthranilic acid organisms showed all the possible combinations in the utilization of anthranilic acid, tryptophan and nicotinic acid.

The oxidation of nicotinic acid is not a general property of pseudomonads of the *Pseudomonas fluorescens* type. A strain of *Ps. pyocyanea* (*Ps. aeruginosa*) oxidized anthranilic acid and tryptophan but not nicotinic acid. Four strains of *Ps. fluorescens* supplied by Dr R. Y. Stanier, isolated, respectively, from benzoate, alanine, glycerol, and succinate enrichments, did not oxidize nicotinic acid; all four strains oxidized tryptophan and only one oxidized anthranilic acid.

The divergence in metabolic pathways underlying the observations that some tryptophan oxidizers utilize anthranilic acid and others do not, is being examined elsewhere (Stanier, Hayaishi & Tsuchida, 1951).

Quinolinic acid as a growth factor

The position of quinolinic acid in both protist and animal is problematic. Certain *Neurospora* mutants have a growth-factor requirement satisfied by nicotinic acid or by much higher concentrations of quinolinic acid (Henderson, 1949; Bonner & Yanofsky, 1949). Quinolinic acid corrects nicotinic acid deficiency in the rat but, again, at much higher concentrations than does nicotinic acid (Henderson, 1949). One explanation of the superiority of quinolinic acid as a growth factor for the 'quinolinic' pseudomonad is that quinolinic acid is in part converted to nicotinic acid and that the unconverted quinolinic acid blocks the subsequent oxidation of this nicotinic acid. Were this so, nicotinic acid might function more effectively as a vitamin in the presence of quinolinic acid. However, quinolinic acid in concentrations 20 times that of nicotinic acid did not inhibit the oxidation of nicotinic acid. Experience in the use of the organism for the assay of quinolinic acid showed that the effect of the presence of nicotinic acid is additive—an indication that both function similarly. Work in progress at Yale indicates that resting suspensions produce measurable amounts of nicotinic acid from quinolinic acid. This suggests that the activity of quinolinic acid depends on its conversion to nicotinic acid, and that endogenously formed nicotinic acid may be less subject to oxidation than exogenous nicotinic acid.

The superiority of 3-hydroxyanthranilic acid over nicotinic acid as a growth factor may similarly depend on its conversion to nicotinic acid at a rate so slow as to allow the nicotinic acid in turn to be converted immediately to an active compound which is not subject to oxidation. Similarly, nicotinamide may be formed from nicotinic acid and not immediately subject to oxidation. Alternatively, the conversion of these compounds to nicotinic acid may be at a site in the organism which is somehow protected against oxidation.

The singular effectiveness of quinolinic acid as a growth factor for this organism stands out in a comparison with another Gram-negative bacterium, *Xanthomonas pruni*, whose growth-factor requirement is satisfied by all the known intermediates in the biosynthesis of nicotinic acid (Davis, Henderson & Powell, 1951). In the instance of *X. pruni*, however, quinolinic acid shows only one-hundredth the activity of nicotinic acid.

The information on the 'quinolinic' pseudomonad does not as yet settle the issue of whether quinolinic acid plays the role of a functional biosynthetic intermediate or that of a metabolic cul-de-sac.

Dr D. M. Bonner's interest in this investigation is gratefully acknowledged.

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Temperature/Growth Relationships of a Thermophilic Actinomycete, *Micromonospora vulgaris*

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SUMMARY: The only thermophilic actinomycetes found in composts made from lawn cuttings were members of the genus *Micromonospora*. The isolates obtained agreed in the main with the description of *M. vulgaris* (Tsiklinsky) Waksman, Umbreit & Cordon. Three strains have been studied in detail. They are true thermophiles, with an optimum range of growth 45–60°, and appear in abundance at the high-temperature phase of the compost, with a profuse production of aerial mycelium and spores. Spore germination is induced by heat activation, 5 min. at 85° securing growth under suboptimal conditions. Thin suspensions of spores in 1 % sucrose or in very dilute nutrient broth withstand 100° for periods of up to 45 min. Only very dense aqueous or saline suspensions are tolerant of these exposures. Surface growth on cellophan bearing a high density of aerial mycelium and spores withstands dry heating at 100° for 150 min. or at 106° for 5 min.

In an extensive study of 480 cultures of thermophilic and thermoduric microorganisms isolated from the dust, grain, hay, etc., as well as from the milk obtained on various dairy farms, Prickett (1928) found that the only true thermophiles were either spore-forming bacilli or actinomycetes. Gordon & Smith (1949), making a taxonomic survey of 216 presumed thermophiles collected from many sources, were able to reduce the sporing bacilli to two main species, *Bacillus coagulans* and *B. stearothermophilus*. The thermophilic actinomycetes have not yet been studied extensively, but from the accounts so far published it would seem that the forms occurring in grass composts belong predominantly to the genus *Micromonospora*. Tsiklinsky (1899) isolated an actinomyces-like thermophile from decaying straw, manure, etc. which she termed *Thermoactinomyces vulgaris*, and which was renamed *Micromonospora vulgaris* by Waksman, Umbreit & Cordon (1939) as the result of finding similar forms on contact slides in dunged composts. Schütze (1908) was the first to describe in detail the development of a thermophilic actinomycete occurring in spontaneously heated clover hay, which he named *Actinomyces monosporus*, and which from his pictures is clearly a species of *Micromonospora*. In their study of the decomposition of the protein and hemicellulose fractions in grass composts, Forsyth & Webley (1948) noted that the extensive chalk-white encrustation of the upper layers of the compost during the high-temperature phase was attributable to the growth of a species of *Micromonospora*.

The present study deals with the thermophilic actinomycete flora obtained from a series of grass composts put up during two seasons in Webley's (1947) composting container, and in collaboration with Dr Webley. All isolates are micromonosporae, have marked features in common, and, apart from a broader temperature range and lack of growth on potato, agree in the main with the description of *M. vulgaris*.

RESULTS

Growth of micromonospora on compost fibres

The visible efflorescence on the fibres during the high-temperature phase can easily be seen by microscopical examination to be due to the aerial hyphae produced by the micromonospora; single, lateral spores can be found in all stages of development along the hyphae, and also occasionally in *Botrytis*-like clusters. It is of interest to note that only the thermophilic micromonosporae produce an aerial mycelium; the mesophilic species are either devoid of aerial growth or produce at most a few sparse branches (Jensen, 1930). The mode of sporulation of *M. vulgaris* will be discussed elsewhere. In practice, the production of an aerial mycelium is an identifying aid in the preparation of enrichment cultures of the organism, which is difficult to isolate except by such means. The conventional dilution methods are seldom successful.

The vegetative filaments are closely attached to the compost fibres and fragments of leaves (Pl. 1, fig. 3), and withstand considerable mechanical shaking without being dislodged. All attempts to isolate the organism on various cellulose media failed, and it seems that the attachment to the fibres and to strips of filter-paper results mainly from the necessity of a support, for the delicate growth, and of aeration for the production of aerial hyphae. It is significant that the micromonosporae appear only in the upper 6 in. layer of the compost.

Technique of isolation. Successful isolations have been made by each of the following procedures:

(1) Small portions of compost showing visible encrustation are distributed in small screw-capped bottles containing 10 ml. of starch Czapek liquid medium, pH 7·2.

(2) The compost is distributed in sterile Petri dishes, and roughly flattened down with disks of sterile filter-paper moistened with starch Czapek solution or mannitol yeast extract.

(3) Small portions of compost are placed on sterile cellophan disks over sterilized compost in Petri dishes, and the whole moistened with mannitol Bacto-tryptone solution.

Incubation is at 60°, and when the typical dry white surface growth appears on the paper after 1–3 days or longer, the organism is purified by plating. The best results were obtained using nutrient (proteose peptone Lab Lemco) agar to which were added 5 % tryptic digest of casein, 0·5 % pea extract, and 1 % soluble starch, pH 7·2 (CPS medium).

High-temperature phase of compost. Table 1 is representative of the results obtained during an August sampling. After the high-temperature stage, fungi and thermoduric eubacteria over-ran the darkened compost, and the temperature continued to decline. A sample taken 14 days later, when the temperature had dropped to 25°, yielded growth after 6 days' incubation at 60° in an enrichment medium. It is clear that the micromonosporae flourish at the peak of the high-temperature phase, which coincides with the most favourable reaction, pH 6·8–7·0.

Table 1. *Isolation of* Micromonospora vulgaris *from compost of varying temperatures*

Compost					
Day sampled	Temperature (°)	Depth sampled (in.)	Moisture content (%)	pH	First appearance of growth at 60° (days)
1	15	Top	89	4·2	7
2	29	1–3		5·0	7
3	50–65	3–6	63	6·2	6
4	51–65	2–6		6·8	2
5	46–64	2–6	83	6·8	1
6	50–55	3–6	75	7·0	2

Growth at different temperatures. Growth does not take place on any medium at 62–80° or at 30°; it is best at 45–60°, and slight to fair at 37°, depending on humidity, aeration, nature of the inoculum and the medium. The organism is, therefore, a eurithermal thermophile in the sense of Imsenecki & Solnceva (1945) and of Gaughran (1947), although it would be regarded as an obligate or stenothermal thermophile by Bergey (1919) and by Gyllenberg (1951). Tsiklinsky recorded 42° as the temperature minimum for growth, but did not state whether for liquid or solid media. In my experience, Murray's (1944) stress on the importance of a water-saturated environment for the production of surface growth of thermophiles on solid media is fully justified. Thus, incubation at 37° may fail in all cases except in shallow layers of liquid media in stoppered vessels, or in poured plates maintained in a moist chamber. Growth at 37° is atypical, almost devoid of aerial mycelium, and frequently non-viable. At 50–60° colonies typically covered with white aerial mycelium are produced in 18–24 hr. in a moist chamber, and may continue to grow for 1–2 days (Pl. 1, fig. 2). But if, after 24 hr. at 60°, a thinly sown plate is placed at 37°, growth may continue for at least a week, resulting in giant colonies with successive rings of aerial mycelium (Pl. 1, fig. 1). This mode of growth is comparable to that of mesophilic streptomycetes and fungi, but not to that of mesophilic micromonosporae (Erikson, 1949). The aerial mycelium of *M. vulgaris* stains with Sudan black and Sudan red IV, using the methods of Erikson (1947).

For the production of aerial mycelium and abundant sporulation, soluble starch (1 %) proved the best addition. The organism is not a marked carbohydrate fermenter, but is characterized by its proteolytic activities. Peptone (1 %), Bacto-tryptone (0·1 %), and a tryptic digest of casein (5 %) are suitable sources of nitrogen. Nitrate nitrogen does not support growth. Casein digest alone often induces autolysis, and, as Forsyth & Webley (1948) have reported, the continuous use of ordinary nutrient (peptone Lab Lemco) agar at 60° may inhibit sporulation. The organism will grow at 55° in a Czapek salt solution containing 1 % soluble starch, 1 % acid hydrolysate of casein (vitamin-free), plus 0·01 % autolysed yeast extract. For maximum growth and rapid recovery after heat treatment, the CPS medium has been used throughout, unless otherwise stated. The pH of all media was 7·2.

Heat activation of spores

Curran & Evans (1945) demonstrated the stimulating effect of 'heat shocking' upon the germinative capacities of different bacterial spores, and also upon their ability to grow, when germinated, under suboptimal conditions. The three strains of *M. vulgaris* which have been studied in detail are all responsive to such treatment. Thus 5 min. at 75°, 85° or 90° will induce growth in 18 hr. at 60° in cotton-wool plugged small tubes of less rich media, which unheated fail to produce growth within the same period—e.g. 4, 3, 2, and 1 % casein digest—Czapek salts liquid media containing 1 % sucrose and 0·01 % autolysed yeast extract. Similarly, the procedure of pre-heating a 250 ml. flask containing 150 ml. 1 % maltose + 5 % casein digest Czapek salts medium to 60° and the inoculum to 85° for 10 min., rapidly sowing the flask, shaking it at 33° for 30 min., and then immediately incubating at 60° gives a more or less coherent surface growth with abundant aerial mycelium in 18 hr.; whereas unheated control flasks, sown with unheated inoculum from the same source, and shaken at room temperature, yield, as a rule, poor bottom vegetative growth only.

After 15 min. at 85° the vegetative mycelium appears to be killed, while the spores for the most part continue to stain well with Gray's spore stain. These spores are not acid-fast. This method, so widely used with other organisms for obtaining an inoculum consisting of spores, has been adopted as a routine.

Preparation of spore suspensions. As with all actinomycetes producing a non-wetting aerial mycelium, considerable difficulty may be experienced in obtaining smooth suspensions. The spores of this organism seem more susceptible to the action of wetting agents than those of mesophilic streptomycetes, and so far one which is non-toxic in effective concentration has not been found. Repeated centrifuging and straining off the flecks of material will ensure a fairly homogeneous suspension, but, if too prolonged, viability is impaired. Filtration by means of positive pressure through three to five layers of sterile filter-paper is less injurious, but wasteful of material. The growth obtained on cellophan placed over the solid medium (Erikson, 1947) has a lower proportion of vegetative to aerial mycelium, and therefore fragments more readily and is more easily suspended after the initial heating than that washed from an agar plate; it is also much more economical of material. The method finally employed was to take the growth on a half-inch cellophan square, rub it off with 5 ml. of the suspending fluid into a centrifuge tube, spin to obtain a fairly even dense suspension, heat at 85° for 15 min., and then dilute, filtering if necessary, so as to yield a suspension containing approximately $15\text{–}30 \times 10^3$ viable cells/ml.

The growth on cellophan, when stored at 2° in a sterile stoppered bottle, retains its vitality for periods up to 6 months. Agar cultures, kept in the refrigerator, may die within a week, although they may remain viable for 1–3 months in stoppered vessels at room temperature. The stock material was kept on cellophan at 2°.

Technique of heat testing. Schütze was disinclined to identify his thermophilic micromonospora with that of Tsiklinsky because his organism withstood 100° for only 5 min., whereas hers resisted for 20 min. In an analysis of the results of the earlier workers on thermobiosis, Hampil (1932) showed very clearly that the degree of thermal resistance depended largely on the experimental methods. Preliminary experiments with unwashed fragments of a surface pellicle of strain M from an 8-day liquid culture, which was not centrifuged, showed that a few spores survived 30 min. boiling in distilled water in cotton-wool plugged tubes. But when thin aqueous spore suspensions of all three strains, prepared as described above, were heated for varying periods at 100° in sealed capillaries according to the technique of Morrison & Tanner (1924), no survivals were obtained. In all experiments, therefore, heating was carried out in sealed capillaries, which were completely immersed in the water-bath.

Effect of suspending fluid upon heat resistance. The work of Robertson (1927) on the effect of osmotic pressure on heat resistance emphasized the protective action of nutrient broth and of sucrose solutions of varying concentrations upon the bacterial cells. This is very marked in the case of the micromonospora spores, which, when adequately dispersed in thin aqueous or saline suspensions, are unable to withstand even 85° for 15 min.

In a series of experiments designed to discover the longevity of the spore suspensions after this preliminary heating, it was found that when aqueous and saline washings were made from growth on an agar plate, centrifuged once, diluted, heated, and plated immediately, only 4–6 % survived. If the suspensions were placed at 2° after heating, 98–100 % of the spores died within 24 hr. Only in very dense suspensions (containing 500–1000 times as many cells as the former) were 1–2 % survivals found after storage at 2° for 6 days. When the experiments were repeated with the surface growth on cellophan strips, which contained a very much lower proportion of diffusible nutrients to be carried over into the suspensions, no survivals were obtained in aqueous or saline suspensions.

Table 2. *Effect of heating broth suspensions of* Micromonospora vulgaris *for 15 min. at different temperatures*

Temperature (°) ...	60	65	70	75	80	85	90	95
Viable cells/ml. ...	21,000	15,500	15,000	19,000	18,000	26,000	16,000	4000

Table 2 shows the protective effect of nutrient broth, diluted 100 times, as the suspending medium. These figures represent the average of six experiments. The colony count rests on the assumption that each colony is derived from a single spore. Frequent examination of slide cultures has shown that this may not always be true; two or three spores may be seen attached to a fragment of mycelium in the most dilute suspensions; and two or three may be capable of simultaneous germination. Such counts can therefore be taken only as approximate.

Similar values were obtained with 1 % sucrose as the suspending fluid; higher concentrations of the sugar were not as effective. These dilute broth and sucrose suspensions could be stored at 2° for 24 hr. without appreciable loss of viability; more prolonged storage resulted in loss. When such suspensions are plated immediately after heating, the stimulating effect of a short period at 85° is discernible. This is more noticeable at 5 min. than after 15 min. and is generally lost when the suspensions are allowed to stand, even in the cold. The spores of 1–7 day cultures grown at 60° behaved in the same way. After 8–15 days the property of heat-resistance declines together with that of viability. No significant differences were found between the three strains.

Thermal death point. For these tests the dilute broth or sucrose suspensions of spores, prepared as described, were placed directly in ice after the preliminary heating at 85°. They were then distributed on 0·2 ml. quantities in capillary tubes, sealed, and immersed in a water-bath at 100° for varying periods of time. When taken out of the water-bath they were plunged into ice, and left there for approximately 1 hr. until all were ready to be plated. The maximum period of resistance to 100° was 45 min., while 30–35 min. was the more usual end-point. Because of the frequent inhomogeneity of the suspensions, even when prepared in exactly the same manner, the experiments were continued on an average three times weekly over a period of 4 months. Table 3 is representative of the results obtained.

Table 3. *Effect of heating sucrose suspensions of* Micromonospora vulgaris *at* 100° *for varying periods*

Time heated (min.) ...	0	5	10	15	20	25	30	35
Viable cells/ml. ...	17,200	6000	2200	400	200	100	100	0

Little difference could be found in the heat resistance of spores of 1–7 day growths, or in that of the spores obtained from 2-day cellophan growths kept at 2° for periods varying from 1 day to 3 months.

Resistance to dry heat. When small strips of the stock growth on cellophan were placed directly into dry capillaries, sealed, and heated in the water-bath, the maximum period for survival at 100° was found to be 150 min. Similar strips in sealed capillaries were then autoclaved, and proved capable of withstanding 5 lb./sq. in. pressure (106°) for 5 min. Duplicate sets of suspensions were prepared from the dry-heated growth, and one set of poured plates incubated at 60° as usual, the other set at 37°. There was little difference in the ratio of survivals at the two temperatures, but the colonies developing at 60° were regularly covered with aerial mycelium, while those grown at 37° had very little.

Effect of heat treatment

On the whole, the growth obtained in all heat-testing experiments has shown remarkable uniformity, being indistinguishable from the stock unheated controls, and showing no loss in vigour or viability. Unheated material was used as the starting-point in all the experiments previously described. When continued subcultures were made from typical colonies derived from heat-treated spores, no progressive enhancement of resistance was obtained.

Occasionally, in about 1 % of these colonies, there were sectors devoid of aerial mycelium. Subcultures from these sectors grew poorly without aerial mycelium, and died within three generations.

Heat resistance of spores attached to substratum mycelium. When the surface growth is scraped and washed off an agar plate or slope, there still remains growing into the medium the major part of the cells of the colonies, i.e. the substratum or vegetative mycelium with its zonate development of lateral spores, which is comparable with the complete growth of a mesophilic micromonospora (Erikson, 1941). Such washed slopes, flooded with 10 ml. saline, were found to withstand the 1–5 min. heating at 100° required to melt the agar and so yield a liquid suspension, which could be diluted for plating purposes. As the cells cannot be separated from the agar, it is impossible to estimate how far this degree of resistance is due to the protective effect of the colloidal material in suspension.

Stationary liquid cultures are slow to produce bottom vegetative growth (5–7 days), and by then it is usually non-viable, even though on microscopical examination spores may be seen. Unheated liquid cultures shaken at 33° for 1–3 hr. and then incubated at 60° may produce a coherent puffball growth of vegetative mycelium with a sparse development of single spores within 18 hr. None of this material has shown the degree of heat resistance exhibited by the typical surface growth with abundant aerial mycelium and spores.

DISCUSSION

The disparity between the visible abundance of micromonosporae in the hot compost and the infrequency with which they appear on dilution plates has been remarked by Waksman *et al.* (1939) and also by Forsyth & Webley (1948). In the present work it has been shown that, if several suitable enrichment methods are adopted, the growth of the organism can constantly be demonstrated although its purification may be a time-consuming matter. It is more readily obtained, because present in larger numbers, in the high-temperature phase, but it can also be isolated when the white efflorescence is no longer visible; e.g. 14 days later, when the temperature has dropped to 25°. Grass cuttings taken in winter (January), and incubated in an enrichment medium at 60° for 18 days have also yielded the same organism.

From the experiments made with aerial growth on cellophan stored at 2°, it is clear that the surface mycelium bearing an abundance of spores is capable of retaining viability and heat-resistant properties for periods up to 6 months. There seems no difficulty, therefore, in postulating that it is by means of its spores that the organism survives outdoors during long periods of temperatures unfavourable for growth.

What is remarkable is the ability of a small proportion of the spores to withstand such prolonged high temperatures as 45 min. at 100° in a dilute sucrose or peptone solution, or 150 min. at 100° when the entire growth is adherent to strips of cellophan simulating compost fibres. Such powers of heat resistance bring the non-acid-fast micromonospora spores in line with certain eubacterial endospores. It is significant that there has been shown a correlation between an abundance of lipid-staining aerial mycelium on the one hand, and vigorous growth at 60° with the production of heat-resistant spores on the other.

This work has been done by the author as a member of the scientific staff of the Agricultural Research Council.

I wish to thank Prof. J. Cruickshank, C.B.E., for the hospitality of his department, and for many interesting discussions on the subject. I am deeply indebted to Dr D. M. Webley for much generous assistance. I also wish to thank Miss Irene Taylor for taking the photographs, Miss Mildred Mackay for invaluable technical assistance, and Dr J. W. Porteous for the pH and moisture content estimations. Grateful acknowledgement is made to the Agricultural Research Council for an expenses grant.

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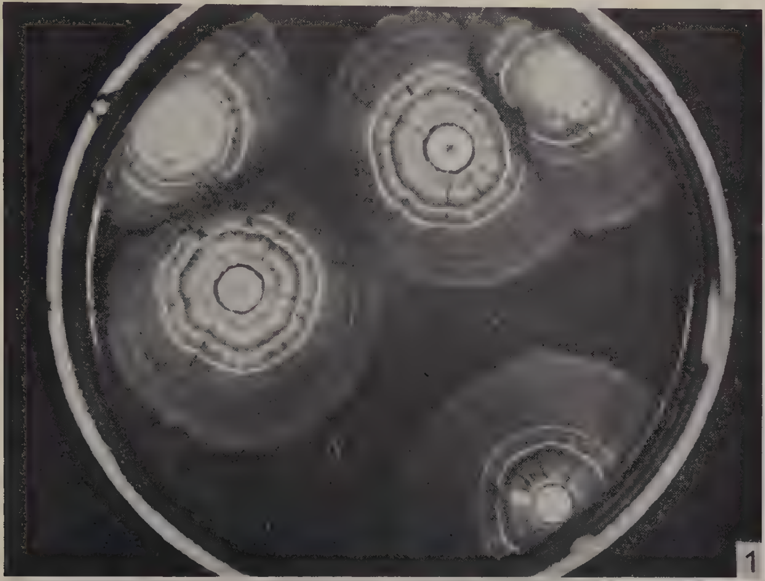
EXPLANATION OF PLATE

Fig. 1. Giant colonies of *M. vulgaris*, showing successive rings of growth with diminishing quantities of aerial mycelium, 1 day at 60° followed by 7 days at 37°. × 1.

Fig. 2. Colonies of *M. vulgaris*, showing regular development of aerial mycelium, 2 days at 60°. × 1.

Fig. 3. Short, sporing filaments of *Micromonospora vulgaris* attached to compost fibre. × 960.

(*Received* 24 *September* 1951)



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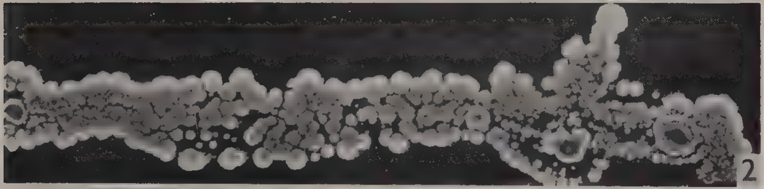

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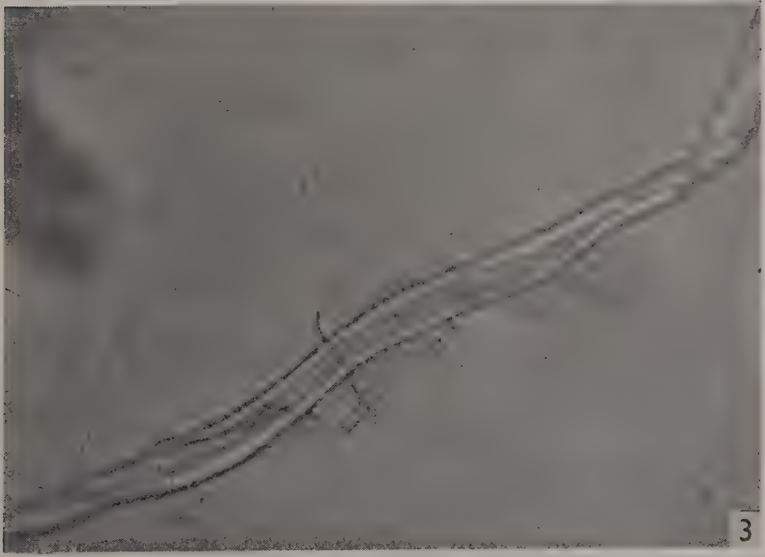

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D. Erikson—Properties of a thermophilic actinomycete. Plate 1

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Antibiotic Production by Fungi on Organic Manures and in Soil

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SUMMARY: *Penicillium patulum, Aspergillus clavatus* and *A. terreus* produced antibiotic substances when grown on organic materials which are used or could be used as manures. The addition of glucose increased the antibiotic titre in every case.

Antibiotics were also formed in autoclaved soils enriched with easily available carbon sources and inoculated with one of the following fungi: *P. patulum, A. clavatus, A. terreus* and two unidentified species of *Penicillium*. *P. patulum* formed an antibiotic also on partially sterilized supplemented soil. The degree of antibiotic activity was decreased when soil cultures of *P. patulum* were contaminated with other organisms from soil or from the air, but even gross contamination with natural soil at 25° did not destroy activity entirely until 11 days had elapsed. Loss of activity was smaller at lower temperatures.

The soil is the natural habitat of the majority of micro-organisms which produce antibiotics when grown on laboratory culture media. Some of these substances inhibit *in vitro* the germination of fungal spores and the growth of certain fungal plant pathogens. If, therefore, the soil flora could be induced to form antibiotics in the soil it might be possible to control soil-borne pathogens very effectively by inhibiting their development in the soil. However, in culture work, abundant antibiotic production necessitates a high level of nutrition and the use of complex media, and it does not follow that even organisms whose natural habitat is the soil can form significant amounts of antibiotics therein, as it is a nutritionally poor medium (Waksman, 1948). The addition of microbial food would therefore seem necessary for antibiotic production, but nevertheless it is known that substances can be extracted from untreated soil which inhibit the growth of micro-organisms (Winter, 1940; Waksman & Woodruff, 1942).

Indirect evidence of antibiotic action in the field is provided by the large number of reports which show that certain soil-borne pathogens may be controlled by the use of organic manures, for example farmyard and various green manures, and straw. Many factors, such as competition for food, accumulation of carbon dioxide (Garrett, 1944), and alteration of pH, are known to contribute to the effect, but antibiotic formation by the developing soil microflora might also hinder the development of pathogens.

Evidence of antibiotic production in supplemented soil cultures was secured by Lewis (1929) using bacteria, and by Nickell & Burkholder (1947) with actinomycetes. Preliminary accounts of work with fungi were given by Grossbard (1947, 1948*a*), who showed that although extracts from autoclaved soil inoculated with *Penicillium patulum* failed to inhibit the growth of certain bacteria and fungi, when the soils were supplemented with carbohydrate the centrifugates from such soils displayed considerable antibiotic activity. Grossbard (1948*b*, 1949, 1950) found that two unidentified species of *Penicillium, Aspergillus terreus* and *A. clavatus*, could also form antibiotics in enriched soil, while Gottlieb & Siminoff (1950*a*) showed that *A. clavatus* was active in this way. Brian, Wright, Stubbs & Way (1951) secured evidence concerning the formation of griseofulvin in the soil by *P. griseofulvum*. The production of antibacterial substances in unsterilized soil supplemented with microbial food, and their presence in the aqueous phase of the soil, has been demonstrated by Nandi (1948).

The present paper deals with antibiotic production by soil fungi, especially *P. patulum*, cultivated on organic materials which are used or could be used as manures, and in soil to which various carbohydrates were added. Grossbard (1945) showed that *A. clavatus* formed antibiotic material when grown on wheat straw. *P. patulum, A. clavatus* and *A. terreus* were chosen for the present investigations because they all produce expansine (synonyms: clavacin, clavatin, claviformin, patulin, penicidin, anhydro-3-hydroxymethylenetetrahydro-1 : 4-pyrone-2-carboxylic acid) in culture media of defined composition. This antibiotic is formed by a wide range of soil fungi and shows, in addition to antibacterial properties, marked antifungal activity. The ultimate object of this work is to establish whether the formation of antibiotics in natural soil by fungi is possible and whether this can be an important factor in the control of certain root diseases by organic manuring.

MATERIALS AND METHODS

Organisms. P. patulum Bainier, strain P. 189, supplied by Mr G. Smith, was used. According to Raper & Thom (1949) *P. patulum* is identical with *P. urticae* Bainier. Two isolates of *A. clavatus* Desm., kindly supplied by Roche Products, Ltd., *A. terreus* Thom, (NCTC 3911) and two unidentified species of *Penicillium* were also used. The latter were isolated by the author from the rhizosphere of tomato seedlings growing in soil infected with *Phytophthora parasitica* but which nevertheless remained healthy.

Manures and soils. The plant materials were harvested in July or September and comprised bracken (*Pteridium aquilinum* Kuhn), red fescue (*Festuca rubra* L.), Italian rye grass (*Lolium multiflorum* Lam.), lawn mowings, white mustard (*Brassica hirta* Moench), red clover (*Trifolium pratense* L.), lucerne (*Medicago sativa* L.), sainfoin (*Onobrychis viciaefolia* Scop.), timothy grass (*Phleum pratense* L.) and wheat straw. Most of these crop plants were supplied by district officers of the National Agricultural Advisory Service; samples of timothy grass and red fescue were supplied by the Welsh Plant Breeding Station. Other materials tested were well decomposed horse manure from animals bedded on wheat straw, and sugar-beet pulp supplied by the British Sugar Corporation Ltd.

Most of the work was carried out on a medium loam subsoil, of a type frequently used for mushroom bed casings. The author is indebted to Dr O. Owen for the following analysis: total nitrogen, 0·41 %; organic carbon

(Walkley & Black), 3·23 %; citric acid soluble potash (as K_2O), 0·029 %; citric acid soluble phosphate (as P_2O_5), 0·015 %; carbonates, slight traces; pH value, 5·52.

Preparation of materials. The manures were sometimes used in the fresh and the decomposed state, fresh materials being cut into short lengths. Quantities (10 g.) were put into 250 or 500 ml. Erlenmeyer flasks with 100 ml. of distilled water and autoclaved at 15 lb./sq.in. for 20 min. In several cases 3·5 % (w/w) of glucose was added before autoclaving.

The soil was passed through a 2 mm. sieve and the water content adjusted to 100 % of the moisture holding capacity, which varied according to the supplement. Portions (100 g.) of soil, alone or supplemented with glucose, sugar-beet pulp or wheat straw, were placed in 250 ml. Erlenmeyer flasks and autoclaved at 15 lb./sq.in. for 1 hr. or placed in an already boiling steamer for 30 min.

Inoculation procedure. The manures were inoculated with a loopful of spores from 1-week slope cultures of the fungi grown on a medium in distilled water and containing (as %, w/w) $NaNO_3$, 0·3; KH_2PO_4, 0·1; KCl, 0·05; $MgSO_4.7H_2O$, 0·05; $FeSO_4.7H_2O$, 0·001; glucose, 4·0; malt extract (Allen and Hanburys Ltd.), 0·2; agar, 1·5.

The soils were inoculated with 10 ml. of spore suspension in distilled water prepared from the above slope cultures; 10 ml. water/slope were used and further diluted 10 times. Controls were set up in a similar manner, omitting inoculation with the fungus.

Extraction of the cultures. Manures and soils were incubated at 25° and usually assayed at weekly intervals. The soil cultures were centrifuged for 45 min. at 2800 r.p.m. (r.c.f. 2260). The fluids obtained, as well as samples pipetted from the manure cultures, were assayed without further treatment.

Assay of antibiotic activity

Antibacterial activity. Bacillus mycoides Flügge and *Bacterium carotovorum* Jones (both supplied by Dr W. J. Dowson) were chosen as test organisms, the former because of its considerable sensitivity to inhibition or stimulation by soil extracts and its ability to show clearly partial degrees of inhibition. The latter was included not only because it was Gram-negative but because, being a plant pathogen, it appeared suitable for use in investigations bearing on the control of soil-borne plant diseases. Occasionally *Bact. coli* (NCTC 86) was used.

The cylinder-plate method was used at first and later the cup-plate method, employing a medium made up in distilled water and containing (as %, w/w) Difco beef extract, 0·3; Difco yeast extract, 0·3; Bacto-peptone, 1·0; NaCl, 0·5; agar, 1·15; pH 6·8.

Yeast extract was incorporated because the zones of inhibition formed by *Bact. carotovorum* on this medium were comparatively well defined. The medium was bulk-seeded from 18 hr. broth cultures of the test organisms, giving a final 1/200 dilution of the inoculum. Expansine was used as a standard for assessing the activity of the test fluids, and the results have usually been expressed in terms of the 'expansine equivalent', the quantity of antibiotic, calculated as expansine, present in a culture. Expansine was dissolved either in pH 6·5 buffer solution or in the centrifugates from the controls in the soil experiments. A standard curve was constructed daily from the readings obtained from a range of concentrations. Each assay plate contained two concentrations of the standard. The unknown was tested in six or ten replicates for each organism. The samples of expansine were given by Boots Pure Drug Co. Ltd. and by Roche Products Ltd.

Antifungal activity. The serial dilution method was used. The medium was pea broth (Leonian, 1934) and the test organisms were the plant pathogens *Ph. parasitica* Dastur and *Thielaviopsis basicola* Ferraris (supplied by Mr P. H. Williams) grown on potato glucose agar. Tubes (6 × 1 in.) containing 10 ml. of medium including the various concentrations of test fluids were inoculated with *Ph. parasitica* by means of 5 mm. disks cut from the most recent zone of growth in cultures 7 days old. The inoculum of *T. basicola* was a loopful of spores. The tubes were examined at intervals of 5 days; the results presented are those obtained after 3 weeks at 25°.

RESULTS

Antibiotic production by Penicillium patulum *on various substrates*

The first point to be decided was whether organic materials which are used or could be used as manures would support antibiotic production. Using wheat straw and timothy grass, the results shown in Fig. 1 were obtained. Here the 'expansine equivalents' are plotted on a logarithmic scale (base 2) against time. Evidently these materials (at least after being autoclaved) are adequate substrates for antibiotic production by this fungus. In both cases production was rapid, and when the first assays were done at the end of a week, the timothy grass culture contained more than twice as much antibiotic as the straw. Between the second and ninth weeks activity in the straw cultures remained constant, after which a slow decline set in. The decline in the timothy grass culture began sooner and was more rapid and after 23 weeks very little activity could be detected, although the straw cultures still showed a moderate degree of activity.

The results of further experiments with a variety of materials, in which trials were also made with the addition of 3·5 % glucose, are shown in Tables 1 and 2. The previous finding that maximal concentrations of antibiotic were frequently present after about 2 weeks, following which there was usually a decline, was confirmed. Accordingly only the results obtained after 14 days are recorded in Tables 1 and 2, in which the expansine equivalents are related to that present in a wheat straw culture, taken as one hundred.

The results show that antibiotic material was produced on several media, the best being sugar-beet pulp. Some media (Table 1) consistently gave the same type of response, i.e. always some antibiotic activity or always no activity at all, whenever tested. Others (Table 2) gave much more variable responses, in that their adequacy or otherwise seemed to depend on the district in which the crop was grown, as is indicated by the results with red clover and rye grass. The time at which the material was harvested may also be important, as is suggested by the data relating to mustard. It is possible that different strains or varieties would have given different results but no evidence to support this hypothesis is available. Generally, the leguminous crop materials were unsuitable, though one of four red clover samples did support the formation of a stable antibiotic. In the others there was some activity at 4 days, which gradually decreased and was seldom detectable after 8–10 days. With unsuitable materials the addition of glucose led to the formation of antibiotic of considerable titre, indicating that the absence of activity was probably due to the lack of a suitable carbon source. The addition of glucose to materials already adequate always caused a large increase in antibiotic titre. It is interesting to note that suitable materials became unsuitable after composting, but became suitable again when supplemented with glucose. Apparently sources of available carbon were removed during the decomposition.

Table 1. *Relative antibacterial activity of* 14-*day cultures of* Penicillium patulum *on substrates giving qualitatively constant results, and the effect of the addition of glucose*

Relative to wheat straw as 100, which here represents an expansine equivalent of 50 mg. Test organism, *B. mycoides.*

	Relative antibacterial activity (%)	
Substrate	Alone	With 3·5 % glucose
Wheat straw, fresh	100	426
Wheat straw, composted	0	391
Lawn mowings, fresh	80	400
Lawn mowings, composted	0	380
Bracken	33	274
Timothy grass	200	426
Sugar-beet pulp	316	—
Lucerne	0	200
Sainfoin	0	54
Horse manure	0	—

No correlation was found between rate of growth and sporulation in the cultures, and antibiotic production. Wheat straw cultures showed comparatively slow growth and sporulation but a high antibiotic titre, while lucerne cultures, though inactive, grew rapidly and spored heavily, as also did active cultures of lucerne + glucose. The rates of decrease of antibiotic activity during continued incubation varied with different substrates, confirming the results shown in Fig. 1. Glucose favoured maintenance of activity for prolonged periods.

Antibiotic production by Aspergillus clavatus *and* A. terreus *on various substrates*

The results of tests with *A. clavatus* and *A. terreus* grown on wheat straw, sugar-beet pulp, timothy and fescue grasses, mustard, lucerne and horse manure are shown in Table 3; only the activities after 14 days' incubation are given. The

Table 2. *Relative antibacterial activity of* 14-*day cultures of* Penicillium patulum *on substrates which gave qualitatively inconsistent results, and the effect of the addition of glucose*

(Activity expressed as in Table 1. Test organism, *B. mycoides*.)

Substrate and sample		Origin and time of harvesting	Relative antibacterial activity (%)	
			Alone	With 3·5 % glucose
Red clover	A	Water meadows, River Lea, Broxbourne. Sept. 1948.	0	—
	B	As A. Sept. 1949.	0	—
	C	Broxbourne, hillside. Sept. 1948.	62	372
	D	Brentwood. July 1951.	0	—
Red fescue	A	Writtle nr. Chelmsford. Sept. 1947. Haulms still green.	0	147
	B	Brentwood. Sept. 1949. Haulms still green.	32	348
	C	Brentwood. July 1951. Aberystwyth strain S. 59. Haulms straw coloured after threshing of seeds.	84	400
	D	Aberystwyth. July 1951. Same strain as in C. Aftermath growth after cutting back panicles.	108	201
Mustard	A	Brentwood. July 1948. Haulms still green.	0	174
	B	Brentwood. Sept. 1948. Haulms straw coloured.	21	300
Rye grass	A	Brentwood. July 1948.	0	—
	B	Broxbourne, hillside. July 1948.	83	—

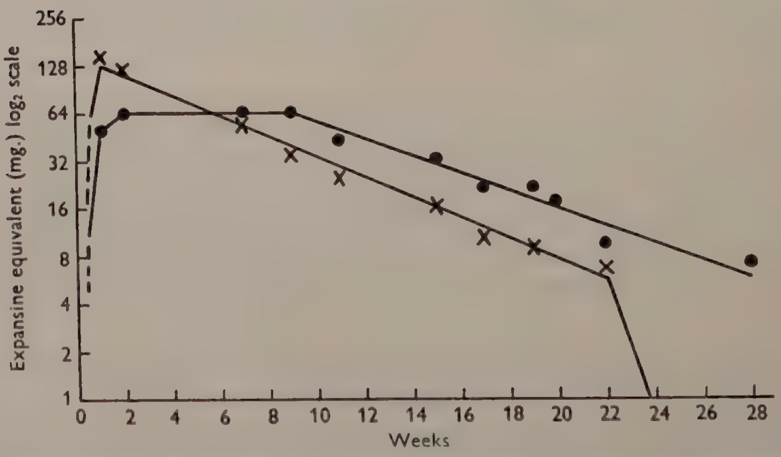

Fig. 1. The antibacterial activity of cultures of *Penicillium patulum* on wheat straw and timothy grass. ×—×, timothy grass; ●—●, wheat straw.

general picture is similar to that with *P. patulum*; sugar-beet pulp, timothy grass and wheat straw are adequate materials in decreasing order of efficiency.

The fluids obtained from cultures of *A. terreus* on wheat straw and timothy grass were active against *B. mycoides* but inactive against *Bact. carotovorum*, whereas *A. clavatus* cultures on all these materials were nearly equally active against both organisms. It was further observed that the zones of inhibition of *B. mycoides* with the *A. terreus* fluids had ragged edges, whereas this organism gives sharp-edged zones with expansine solutions (Pl. 1, figs. 1, 2). Although *A. terreus* does form expansine (Kent & Heatley, 1945) the above observations suggest that it is not formed on wheat straw or timothy. However, cultures on wheat straw with added glucose, and on sugar-beet pulp, were both slightly active against *Bact. carotovorum* and possibly expansine was present therein, suggesting that expansine formation depends even more strongly with *A. terreus* than with *A. clavatus* on the presence of easily available carbohydrate.

Table 3. *The antibacterial activity of cultures of* Aspergillus clavatus *and* A. terreus *growing on different substrates*

	Expansine equivalent (mg.)			
	A. clavatus		*A. terreus*	
Substrate	*B. mycoides*	*Bact. carotovorum*	*B. mycoides*	*Bact. carotovorum*
Fresh wheat straw	28	30	11	0
Fresh wheat straw + 3·5 % glucose	110	117	38	6
Sugar-beet pulp	49	50	21	3
Timothy grass	40	41	13	0
Fescue	0*	0*	0	0
Mustard	0*	0*	0	0
Lucerne	0	0	0	0
Horse manure	0	0	0	0

* Results were variable: low antibiotic activity was occasionally observed on these substrates.

Antibiotic formation by Penicillium patulum *in autoclaved soil*

Grossbard (1947, 1948*a*) showed that while unsupplemented autoclaved soil did not support antibiotic production by *P. patulum*, the addition of energy-yielding materials led to a positive response. Therefore the ability of sugar-beet pulp and wheat straw to support antibiotic production by *P. patulum* when incorporated in soil at various concentrations and the mixtures autoclaved, was compared with that of soil + glucose. In addition, comparisons were made between completely untreated soil, autoclaved but unsupplemented and uninoculated soil, and autoclaved unsupplemented but inoculated soil. Assays were made after 14 days, against both *B. mycoides* and *Bact. carotovorum*.

Evidently, for antibiotic production to occur the substrate must be autoclaved and inoculated with the fungus (Pl. 1, figs. 2, 3, *e*, *h*, *i*). No antibiotic was produced unless these conditions were fulfilled (*b*, *c*, *d*, *f*, *g*). However, *B. mycoides* was stimulated by materials in the centrifugate from untreated soil, and this effect was increased when the soil was autoclaved (Pl. 1, fig. 2, *b*, *c*). There was no further response when non-supplemented autoclaved soil was inoculated (Pl. 1, fig. 2, *d*). When the soil was supplemented with sugar-beet pulp but not autoclaved a slight lytic effect, as yet unexplained, was observed (Pl. 1, fig. 2, *f*). This disappeared on autoclaving, but the zone of stimulation was larger (Pl. 1, fig. 2, *g*). *Bact. carotovorum* was not stimulated by the unsupplemented soil centrifugates which stimulated *B. mycoides*, as shown in Pl. 1, fig. 3, *b*–*d*, but there was some stimulation with sugar-beet pulp (Pl. 1, fig. 3, *f*, *g*).

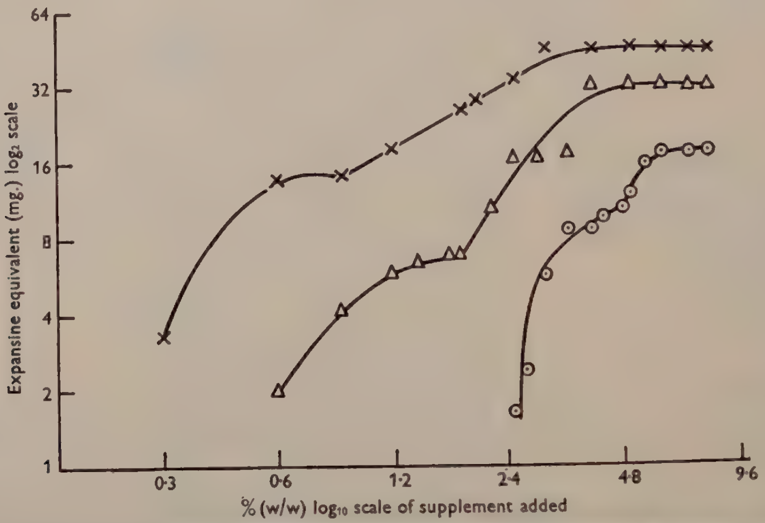

Fig. 2. Antibacterial activity of centrifugates from autoclaved soil receiving various supplements and inoculated with *P. patulum*. ×—×, glucose; △—△, sugar-beet pulp; ⊙—⊙, wheat straw. Test organism, *B. mycoides*.

As shown by Grossbard (1948*b*) centrifugates from certain untreated soils did occasionally give narrow zones of partial inhibition not only of *Bact. coli* but also of *Bact. carotovorum*. In the present work, very large zones of weak partial inhibition of *Bact. carotovorum* were obtained with centrifugates from glucose supplemented but uninoculated soils; occasionally this effect was observed even with *B. mycoides*.

The quantitative data of this experiment are given in Fig. 2. The expansine equivalents have been plotted against the amount of material added (logarithmic scales in both cases), and it can be seen that weight for weight glucose was more effective than sugar-beet pulp, wheat straw being still less valuable, although effective when in sufficient amount. Three features of the curves are striking. First, there was a threshold quantity necessary for appreciable antibiotic formation with each material, the amounts varying from 0·3 % with glucose to 2·5 % with wheat straw. Secondly, maximum production, which reached different levels in the three cases, was attained over a much narrower range, the order being the same as with the threshold quantities. Thirdly, each of the graphs relating $\log_{10}$ concentration to $\log_2$ expansine equivalent shows a 'two-stage' effect; apparently a first maximum in production was reached with a relatively small amount of each material, with subsequent further stimulation of activity when larger amounts were added. The reason for this is at present obscure. It may be an artefact due to the cycle of increase and decrease in antibiotic (Fig. 1) having differed in duration with the different rates of addition of each supplement. If the effect is real, it might be ascribed to the production of two antibiotics at different rates and with different threshold levels and maxima, so that in reality two antibiotic production curves are superimposed.

Table 4. *The antifungal activity of centrifugates taken from autoclaved soils receiving 2·5 or 5 % (w/w) of various supplements, and compared with their antibacterial activity*

	pH value		Highest dilution giving complete inhibition of			
Centrifugates from	Before incubation with *P. patulum*	After incubation with *P. patulum*	*Ph. parasitica**	*T. basicola**	*B. mycoides*†	*Bact. carotovorum*†
Soil only	5·25	5·89	Stimulation	Stimulation	Stimulation	No activity
Soil + glucose 2·5%	5·63	4·93	1/100	1/100	1/40	1/40
„ „ 5·0%	5·23	4·55	1/100	1/100	1/40	1/40
Soil + sugar-beet pulp 2·5 %	4·77	4·94	1/80	1/40	1/30	1/30
„ „ „ „ 5·0 %	4·96	4·84	1/100	1/80	1/40	1/40
Soil + wheat straw 2·5 %	5·03	4·86	1/10	1/5	—‡	1/5
„ „ „ 5·0 %	5·02	5·25	1/20	1/10	1/10	1/20

* For 3 weeks at 25°.
† For 18 hr. at 25°.
‡ Partial inhibition only at 1/5.

The fluids obtained from these supplemented soils, autoclaved and inoculated with *P. patulum*, also possessed considerable antifungal activity as shown in Table 4, which gives the dilutions of the soil centrifugates at which complete inhibition of *Ph. parasitica* and *T. basicola* occurred, the soils having received additions of 2·5 or 5 % of glucose, sugar-beet pulp or wheat straw. Glucose was the most potent substance, followed closely by sugar-beet pulp, with wheat straw much less effective. Table 4 also shows the dilutions at which the centrifugates were inhibitory for *B. mycoides* and *Bact. carotovorum*. The antifungal activity was higher than the antibacterial. Expansine shows this type of activity.

Antibiotic production by various fungi in autoclaved supplemented soils

The property of forming an antibiotic in autoclaved supplemented soils is not restricted to *P. patulum*. In Fig. 3 *P. patulum* has been used as a standard for assessing the activities of *A. clavatus*, *A. terreus* and two unidentified species of *Penicillium* in soils supplemented with glucose, sugar-beet pulp or wheat straw.

The pattern of relative activities varied with the different substrates and test organisms used, but *A. clavatus* and *Penicillium* species A were about equally efficient and slightly less active than *P. patulum*; next came *A. terreus*; least active was *Penicillium* species B which was inactive against *B. mycoides* on soil + straw and against *Bact. carotovorum* on all substrates. In soil supplemented with wheat straw or sugar-beet pulp, *A. terreus* failed to yield an antibiotic completely inhibiting the growth of *Bact. carotovorum*, though in the latter case good partial inhibition of that organism was obtained. Fig. 3 does not, however, include partial inhibitions. In experiments not involving soil, which were not intended as true comparisons, *A. clavatus* proved only a little more than half as active as *P. patulum* (Table 3). However, in the present experiment a different isolate was used.

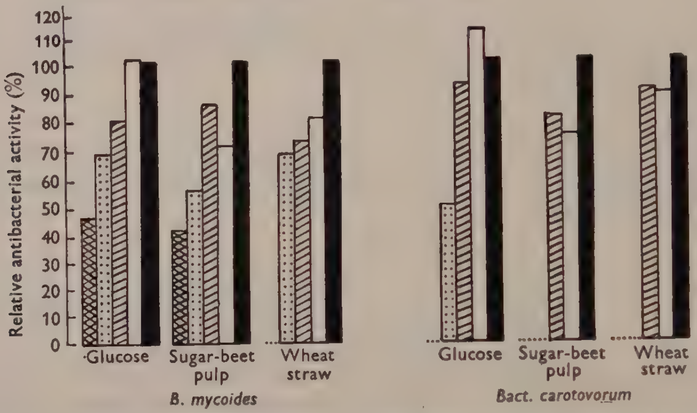

Fig. 3. The relative antibacterial activity of centrifugates from autoclaved soils receiving various supplements and inoculated with five different fungi. ▨, *Penicillium* sp. A; ▩, *Penicillium* sp. B; □, *Aspergillus clavatus*; ⬚, *A. terreus*; ■, *P. patulum*.

Antibiotic production by Penicillium patulum *in soil containing other micro-organisms*

The work described above shows that various fungi can form considerable quantities of antibiotics when grown in pure culture in supplemented and autoclaved soil. The results cannot, however, be directly related to practical conditions for the reason that the soil and the supplements were both subjected to autoclaving, which eliminates the normal flora and may have broken down the organic matter of the soil and of the supplements, causing possibly an enhanced availability of nutrients. In glasshouse practice steaming is used and moderate release of nutrients could occur from both the soil and the supplements, while part of the microflora of the soil would remain to multiply and compete with the antibiotic producer. In the field full competition by the soil flora would occur and there would be no comparable release of nutrients, except by microbial action. Experiments were therefore carried out to study antibiotic production in non-sterile supplemented soil and in steamed supplemented soil, both inoculated with *P. patulum*. The supplements were 2·5 or 4·0 % of sugar-beet pulp or wheat straw, with glucose as a standard.

In no case was any antibiotic detected in the non-sterilized supplemented soils. In Table 5 a comparison is given between the results for steamed and autoclaved soil, similarly supplemented.

Table 5. *A comparison of the antibacterial activity of centrifugates from supplemented steamed or autoclaved soils, prepared* 14 *days after inoculation with* Penicillium patulum

The soil used was different from that used in previous experiments: it was a light loam top soil with a large quantity of plant residue.

	B. mycoides, expansine equivalent (mg.)			*Bact. carotovorum*, expansine equivalent (mg.)		
Centrifugates from	Steamed	Autoclaved	Diminution in yield (%)	Steamed	Autoclaved	Diminution in yield (%)
Soil + glucose 2·5 %	35	55	36	35	57	39
„ „ 4·0 %	36	56	36	37	57	35
Soil + sugar-beet pulp 2·5 %	17	30	43	21	35	40
„ „ „ „ 4·0 %	31	50	38	30	50	40
Soil + wheat straw 2·5 %	0*	4	100	0	5	100
„ „ „ 4·0 %	0*	12	100	0	13	100

* Growth of *B. mycoides* was stimulated.

The steamed mixtures of soil and sugar-beet pulp or glucose supported antibiotic production by *P. patulum*, but activity was lower than when they were autoclaved. This may have been due to competition by the surviving microflora or to lower concentrations of nutrients released by steaming as compared with autoclaving. It is clear, however, that competition from soil organisms which survive the steaming process was not effective in suppressing antibiotic formation in the soil to any great extent, except in the soil-straw mixtures, which were wholly inactive.

Persistence of antibiotic after contamination of cultures on autoclaved soil

Waksman (1950) stated that even if antibiotics were formed in the soil they would not persist owing to destruction by other micro-organisms. However, Nandi (1948), Sinha (1950), Nimkar (1950) and M. Monib (private communication) showed that certain purified antibiotics when added to non-sterile soil did persist for varying periods during which they exerted an influence on the soil microflora. Jefferys (1950) and Gottlieb & Siminoff (1950*a*) also found evidence of antibiotic persistence in soil. These authors used expansine in addition to several other antibiotics.

In order to study the effect of a freshly introduced microflora on the titre of an antibiotic in soil, Erlenmeyer flask cultures of *P. patulum* on autoclaved soil + 2·5 % of glucose and in which the antibiotic had already been formed, were inoculated (contaminated) with other micro-organisms. This inoculation was effected either by adding 20 % of non-sterilized soil of the same type and mixing it with the cultures in the flasks and re-plugging, or by transferring soil from the flasks into open jam jars. The latter were placed under bell jars in which the atmosphere was saturated with water vapour to eliminate evaporation. Each of the two sets was divided into two lots, one being kept at room temperature (mean value 15°) and the other in an incubator at 25°. Another set of flasks, not contaminated, served as a control and was kept in an incubator at 25°. The various activities are recorded in Table 6.

Table 6. *Relative antibacterial activities of sterilized soil containing antibiotic and the same subsequently contaminated with other organisms*

		After exposure to contamination for (days)			
		5	8	11	14
Source of contamination	Temp.	Relative activity (%)*			
None	25°	100	85	74	13
Air	15°†	92	55	85	62
Non-sterile soil	15°†	42	33	20	2
Air	25°	37	12	29	0
Non-sterile soil	25°	30	25	0	0

* Expressed as a percentage of the expansine equivalent at the start, which was 60 mg. by *B. mycoides assay*.

† Mean value during the experiment.

After 5 days the control still showed the initial activity, but subsequently there was a slow fall, and after 14 days comparatively little activity was left. Cultures exposed to the air and left at room temperature behaved differently, the activity declining a little after 5 days, but markedly in the next 3 days and then increasing again to a high level, followed by a slow decline. At 14 days, activity was higher than in the not contaminated cultures. However, no great significance should be attached to this because in previous experiments activity was lost on exposure to air at the same time as in the controls. Exposure at 25° led to a similar pattern of changes but at a lower level as the decline after 5 days was greater, and the antibiotic content fell to zero after 14 days' exposure. Contamination with soil, whether at room temperature or at 25°, caused a large decrease in activity in 5 days; subsequently there was a further slow decline in each case. At 25° no activity was detected after 11 days, but at room temperature the decline was slower and some activity was still present even after 14 days. The irregularity of the fluctuations makes interpretation of the results difficult. However, it is clear that even gross contamination with soil did not lead to immediate destruction of all antibiotic activity. Temperature appeared to have some effect, as at 25° the decreases in activity were larger and more rapid.

DISCUSSION

The fungi *P. patulum*, *A. clavatus* and *A. terreus* can produce antibiotic material on various organic manures but fail to do so on others. Suitable materials, when composted, became unsuitable as substrates for *P. patulum* for antibiotic production. However, the addition of glucose to composted manures and to materials inadequate even in the fresh state rendered them suitable, and it also increased the titre of cultures of all fungi on adequate manures. These results suggest that available carbohydrate is the prime requirement for antibiotic production on these substrates. Soil itself, at least after autoclaving or steaming, contains all the nutrients required for antibiotic production by the fungi tested except energy-providing materials, as shown by the production of antibiotics in such soils after addition of glucose.

Whether unheated soil also contains sufficient nutrients apart from energy sources cannot be stated at present. The failure to obtain antibiotic production in non-sterile soil supplemented in various ways may have been due to lack of nutrients, to competition by the soil flora, or to failure to detect the low concentrations of antibiotic to be expected in a natural soil. To decide the first point, experiments on sterilization of the soil by a method which should not lead to the release of nutrients are now in progress, namely by the use of propylene oxide. Preliminary results have shown that antibiotic formation takes place in glucose supplemented soil, treated with propylene oxide, but with lower yields than in heated soil. From this, one may conclude that soil may be adequate for antibiotic production, provided an energy source is supplied in addition.

Competition by the soil microflora may be an important factor in the limitation of antibiotic production by suppressing the growth of the antibiotic-producing organism or by inhibiting antibiotic production. However, not every type of microbial competition can entirely prevent antibiotic production. This is shown by the experiments with steamed soil + glucose and steamed soil + sugar-beet pulp mixtures. Although part of the original microflora must have survived, at least in the form of spores, antibiotic production took place, though with lower yields. No antibiotic was formed in the steamed soil + straw mixture. Steamed straw, without soil, was an unsatisfactory substrate, antibiotic production being very irregular. Sufficient nutrients may not have been available, and possibly the surviving flora was somewhat different from that on sugar-beet pulp. These suggestions receive support from the results of preliminary work with propylene oxide. While sugar-beet pulp is easily sterilized by this substance and the titre of antibiotic formed by *P. patulum* is almost as great as on autoclaved sugar-beet pulp, it is difficult to achieve complete sterility of wheat straw with propylene oxide. The cultures frequently failed to yield antibiotic, or only low yields were obtained. However, when glucose was added to straw treated with propylene oxide, antibiotic of high concentration was formed readily.

The microflora might also act by gradually destroying antibiotic. This conclusion is in line with the results of the contamination experiment and with the report of Krebs (1944) on the existence of an enzyme which hydrolyses expansine. However, Jefferys (1950) and Gottlieb & Siminoff (1950*b*) showed that expansine persists for a considerable time when added to non-sterile soil. It is possible, therefore, that in non-sterile soil local concentrations of antibiotic occur in the vicinity of energy-yielding particles, as suggested by Brian *et al.* (1951), and that organic manuring, by increasing the content of energy-yielding matter, induces antibiotic production.

In this connexion it is significant that a parallel exists between the requirements essential for antibiotic production by the fungi tested and the conditions necessary for the control of certain root diseases. The fact that some plant materials were suitable, while others, e.g. certain samples of red clover or rye grass were unsuitable, for antibiotic production, fits the results of West & Hildebrand (1941) and of Rouatt & Atkinson (1950), that while soybeans were effective, red clover and rye grass were ineffective in controlling certain plant diseases. Further, abundant carbohydrate must be present if an antibiotic is to be produced by the tested fungi. This can be correlated with the finding of Nandi (1948) who succeeded in extracting from non-sterile soil reinforced with glucose material which showed marked antibacterial properties, and with the evidence in the literature (Garrett, 1938; West & Hildebrand, 1941; Blair, 1943; Grossbard, 1946, 1949; Rouatt & Atkinson, 1950) that soil supplements high in carbohydrate are effective in the control of some root diseases. These effective materials are also low in nitrogen, and work to be published later suggests that increase of nitrogen concentration decreases antibiotic production by *P. patulum* in soil. Thus the results showing the controlling effect of carbohydrate manuring on certain soil-borne diseases should not be entirely dissociated from the possibility that this treatment resulted not only in a modification of the composition of the microflora but also in a formation of local concentrations of antibiotics, perhaps only temporary, which may have exerted an inhibitory action on plant pathogens in the neighbourhood of the roots of host plants.

As authority for the names of plant materials, L. H. Bailey's *Manual of Cultivated Plants*, (1949) and for the bacteria W. J. Dowson's *Manual of Bacterial Plant Diseases* (1949) have been used.

I wish to thank the Agricultural Research Council for a grant which enabled this work to be carried out. A large proportion of the work was done at the Experimental and Research Station, Cheshunt, under the guidance and advice of Dr W. F. Bewley, to whom I am greatly indebted. I wish to thank Dr S. E. Jacobs for his guidance, valuable suggestions and for his help in preparing this paper. Technical assistance, by Mrs D. Sapsford, Miss M. Beadon, Miss C. Liss and Mr H. Tooley who prepared the photographs, is gratefully acknowledged.

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EXPLANATION OF PLATE

(Magnification ×$\frac{2}{3}$)

Fig. 1. Assay of the culture fluid of *A. terreus* growing on wheat straw, using *B. mycoides*. Note diffused edges of the zones with the rhizoid outgrowths of the colonies protruding into the area of inhibition: this picture does not alter, the areas of inhibition do not become overgrown.

Fig. 2. Assay of centrifugates from a soil receiving various treatments. Test organism: *B. mycoides*. *a*, expansine in a buffer solution, 1 g./l. *b*, completely untreated. Note stimulation. *c*, not supplemented, not inoculated, but autoclaved. Note increase in zone of stimulation. *d*, not supplemented, but autoclaved and inoculated with *P. patulum*. Centrifugate prepared 14 days after incubation at 25°. *e*, supplemented with glucose and inoculated as in *d*. *f*, supplemented with sugar-beet pulp but not autoclaved or inoculated. *g*, as *f* but autoclaved. Note increase in zone of stimulation. *h*, as *g* but inoculated with *P. patulum*. Note distinct wide zone of stimulation following the zone of inhibition. *i*, as *h* but supplemented with wheat straw.

Fig. 3. As fig. 2, but the test organism is *Bact. carotovorum*. Zones of stimulation are generally slight or absent. Arrangement of centrifugates as in fig. 2.

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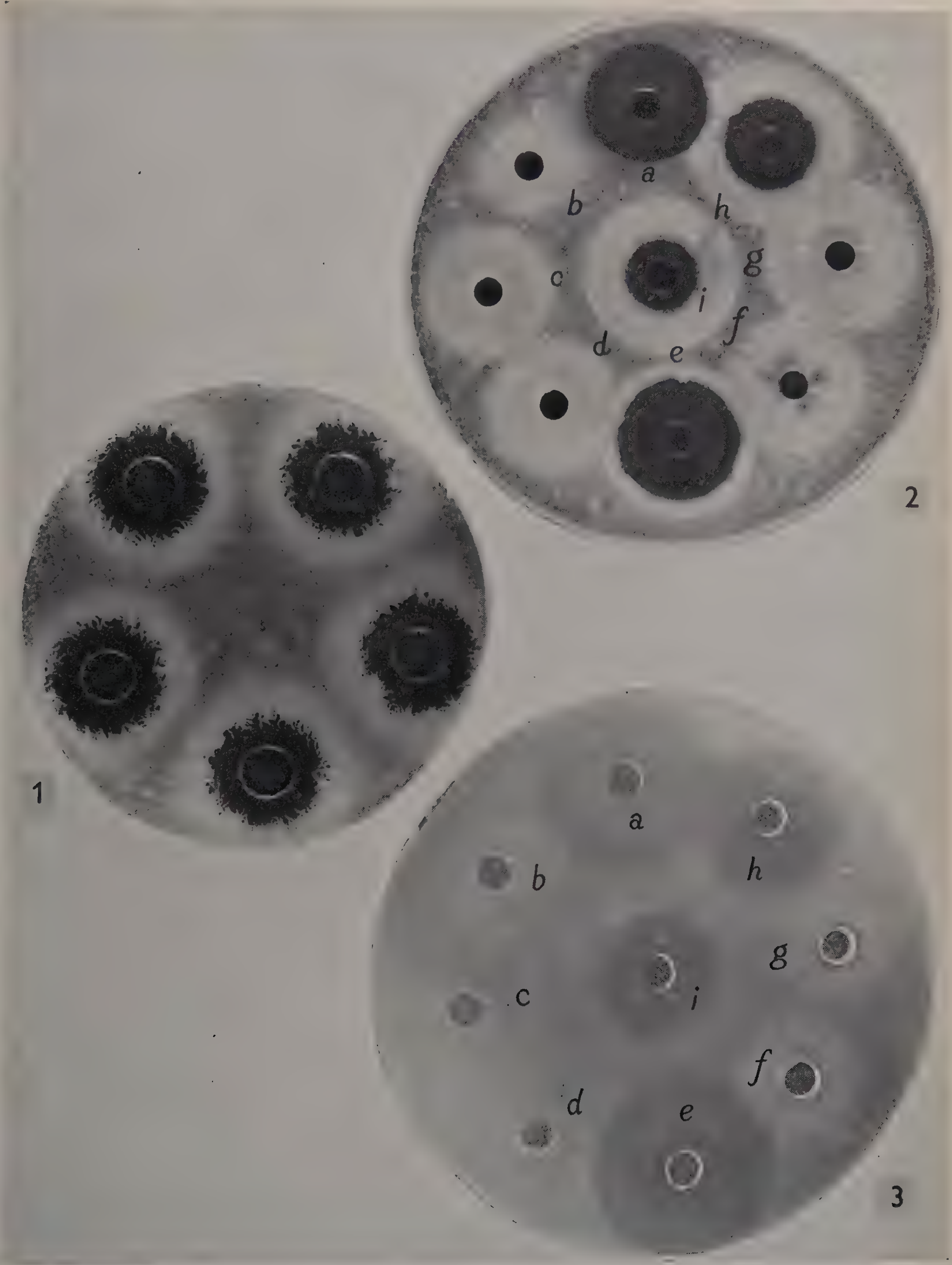
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E. Grossbard—Antibiotic production by fungi in soil. Plate 1

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The L-Form of *Haemophilus pertussis* in the Mouse

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SUMMARY: An examination of Giemsa-stained preparations of lung tissue and peritoneal fluid from mice inoculated with *Haemophilus pertussis* shows that the bacillary form can change to the L-form of the organism *in vivo*. This transformation is more rapid and more complete in immune than in normal mice. The L-form, produced on Bordet-Gengou agar containing 1·5 % (w/v) glycine, administered intranasally can revert to the bacillary form *in vivo*.

Only two reports have been found in the literature on the isolation of L-forms of pathogenic bacteria directly from an animal or from a human being. The first (Klieneberger, 1938) was the L-form of *Streptobacillus moniliformis*, Strain '59 lung' found as an independent organism (not associated with a bacillus) in the lung lesions of a rat. At the time of this report, Klieneberger described the organism as the L1 'symbiont' of *Strep. moniliformis*, but she has since changed her views and now regards the L1 as a growth phase of the bacillus (Klieneberger-Nobel, 1949). The second (Dienes & Smith, 1944) was the L-form of a *Bacteroides* sp. strain 'O.H.', 'cultivated from pus accumulated in the peritoneal cavity following infection of the Fallopian tubes'. However, the authors themselves were in some doubt whether this organism was really in its L-form in the host, and they discuss their reasons for and against believing that the L-form was present in the pus.

In order to gain an understanding of the significance of the L-form, one of the many questions that must be answered is, 'Does the L-form of pathogenic bacteria occur *in vivo* with any degree of regularity, and if so under what conditions?' The present study was undertaken in an effort to obtain an answer to this question.

MATERIALS AND METHODS

Mice. All mice used weighed between 17 and 20 g. and were chosen from an English strain, 'Cl.' or an American strain, 'N.I.H.'.

Strains. A smooth strain of *Haemophilus pertussis*, No. 19939, with a high mouse virulence, was used for infection and challenge of mice. Various smooth strains of *H. pertussis* were used for immunization.

Preparation of suspensions. The bacillary form was grown on 30 % horse blood Bordet-Gengou (BG) medium and the L-form on BG containing 1·5 % (w/v) glycine. The 20 hr. growth was scraped off into 1 % Bacto-Casamino acids and suspensions were standardized against Brown's Opacity Tubes (Burroughs Wellcome and Co. Ltd., London) assuming that *H. pertussis* was equivalent to *H. influenzae*.

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Mice chosen for autopsy. During the first 3 days after inoculation, mice that were apparently well were chosen at random. After the majority of mice had begun to show some signs of illness, the ones that appeared most ill were chosen. Dead mice were not examined because of the presence of contaminants in their tissues.

Control uninoculated mice were also killed and examined for the presence of organisms by the methods used for inoculated mice.

Preparation and cultivation of autopsy material. Immediately after killing a mouse, a portion of its lungs was grasped in a pair of forceps and dragged back and forth over the whole surface of several BG plates, the tissue being squeezed to express some of the fluid. Peritoneal fluid was examined by opening the peritoneal cavity and rinsing with a few drops of 1% Bacto-Casamino acids. The fluid was collected in a capillary pipette and spread on BG plates. Plates were incubated at 35°.

Autopsy material and subcultures from autopsy material were never planted on media containing glycine, penicillin, nor any other substance that might stimulate the production of L-forms.

Preparation of slides. Coverslip impression preparations were made from squares of agar cut from the plate cultures immediately after inoculating the plates (0 hr.), and after 3–5, 24, 48 hr., and sometimes 72 hr. of incubation. The 3–5 hr. preparations were useful in the detection of contaminations which were often overlooked in 0 hr. preparations. Since it is essential in experiments of this type that all preparations be absolutely free of contaminating organisms, all contaminated cultures were immediately discarded. The 48–72 hr. preparations acted as a check on the 24 hr. Furthermore, adequate growth for agglutination tests could often be obtained only after 48 hr. incubation. The impression preparations were fixed, treated, and stained with Giemsa according to methods already described (Klieneberger-Nobel, 1950; Wittler, 1951).

EXPERIMENTAL

Intranasal inoculation of normal mice with the L-form

Mice, inoculated intranasally with the L-form of *H. pertussis* (produced by growth on BG-glycine agar), were killed at various times between the second and twenty-fourth day following inoculation. Lung cultures and impression preparations were made. All except one of the inoculated mice yielded bacilli and occasional small round red forms. Even the 0 hr. preparations, although there were always at least a few L-bodies present, showed predominantly blue bacillary forms. However, one mouse, killed on the 14th day after inoculation, yielded L-forms. Its lungs showed patches of consolidation, but their gross appearance was in no way distinct from any that had been encountered earlier. The 0 hr. impression preparations showed that all the organisms were in the granular stage of the L-cycle (Pl. 1, fig. 1) or in the amorphous stage consisting of shapeless cytoplasmic masses without definite cell walls, in which the nuclear material was dispersed in a variety of patterns. The 3 hr. preparations showed a slight increase in the amount of L-material (Pl. 1, fig. 2) and the

24 hr. ones a very great increase (Pl. 1, fig. 3), but still no bacilli could be found. The 48 hr. Giemsa-stained preparations showed masses of amorphous L-material among which were patches of dark red or purple bodies that were dividing up into bacilli (Pl. 2, fig. 4), as well as many patches of large purple or very pale blue bacilli.

Subcultures made from the 24 hr. growth to fresh BG medium followed the same developmental pattern as the original plates, and after 48 hr. of incubation bacilli were appearing in masses of amorphous L-material (Pl. 2, fig. 5). The second subculture, made from the 72 hr. growth of the first subculture, showed an almost pure growth of the bacillary form which agglutinated strongly with undiluted Phase 1 pertussis antiserum (Pl. 2, fig. 6). This culture was then put through five serial passages on ordinary BG. It remained completely in the bacillary form. When inoculated intranasally into mice, it had the same virulence as the mother culture (bacillary) from which it had originally been derived.

The results of this experiment offered two possible explanations. Either the L-form when injected into mice (*a*) always reverted to the bacillary form, but on occasion could change back into the L-form again, or (*b*) usually reverted to the bacillary form, but on occasion could remain in the L-form. The next step was to investigate whether the bacillary form could change into the L-form *in vivo*.

Intranasal inoculation of normal mice with the bacillary form

Normal mice were inoculated intranasally with bacilli in doses roughly equivalent to the dose of L-forms given in the previous experiment. Mice were killed at various times between 2 hr. and 15 days. Portions of the lungs were wiped over BG plates and impression preparations taken. The following observations were made.

Mice killed 2–4 *hr. after inoculation.* 0 *hr. preparations.* Most of the organisms were blue bacilli in an extracellular position. Occasional small round red forms were present in phagocytic cells. 24 *hr. preparations.* Heavy bacillary growth. No evidence that the organisms had passed through the stages of the L-cycle.

Mice killed 1–2 *days after inoculation.* 0 *hr. preparations.* Some showed the same results as the mice killed 2–4 hr. after inoculation. Others showed a great many small round red forms in phagocytic cells. 24 *hr. preparations.* Heavy bacillary growth. Some organisms which were intracellular or else located in small colonies overgrowing phagocytic cells were in the various L-forms and stained either bright red or very pale blue.

Mice killed 6–7 *days after inoculation.* 0 *hr. preparations.* Most organisms were red, pleomorphic or granular forms located in phagocytic cells. Extracellular organisms were usually blue bacilli. 24 *hr. preparations.* Same as the 1–2 days, 24 hr. preparations except more L-forms present.

Mice killed 12–15 *days after inoculation.* 0 *hr. preparations.* Organisms observed were all intracellular, and were in tissue cells as well as phagocytic cells. They were either small round red forms or pale blue pleomorphic forms.

There was much pale blue or bright red amorphous material in the background which could not be identified with certainty as L-material. *24 hr. preparations.* Many L-forms, large bodies, and small round red forms present in and around cells and tissues. Heavy growth of red or blue bacilli as well.

The number of L-forms appeared to increase with the duration of the infection, which suggested that the change to the L-form might be taking place as a response to an environment that was becoming increasingly less favourable. Since Dienes, Weinberger & Madoff (1950) had shown that, at least *in vitro*, various strains of Salmonella changed into the L-form when exposed to immune serum and complement, it seemed possible that the development of immunity in the host might be the stimulus inducing the transformation of *H. pertussis in vivo.*

Intranasal inoculation of immune mice with the bacillary form

Mice were immunized intraperitoneally with living or formalized *H. pertussis* bacilli with doses ranging from 2000×10^6 to 80×10^6 organisms/mouse. After a rest period of 10 days to 5 weeks, mice were challenged intranasally and were killed at various times between 2 hr. and 18 days thereafter. Portions of lung were wiped over BG plates and impression preparations made as before. The majority of the normal control mice which were challenged died within 3 to 7 days.

Mice killed 2–4 *hr. after challenge.* 0 *hr. preparations.* Most of the organisms were blue bacilli in an extracellular position. Occasional small round red forms were present in phagocytic cells. 24 *hr. preparations.* Heavy bacillary growth. No evidence that the organisms had passed through the stages of the L-cycle.

Mice killed 1–2 *days after challenge.* 0 *hr. preparations.* Nearly all the organisms found were located intracellularly. Red organisms were small round forms, granules, or minute bacilli. Blue organisms were very pale and very pleomorphic. 24 *hr. preparations.* Many stages of the L-cycle present intracellularly and extracellularly. Numerous blue, red, and purple bacilli present extracellularly.

Mice killed 4–7 *days after challenge.* 0 *hr. preparations.* All organisms were in the granular or amorphous stages of the L-cycle and located both intracellularly and extracellularly. 24 *hr. preparations.* All stages of the L-cycle were still present. A few bacilli were appearing at the periphery of masses of amorphous L-material. (Photographs of preparations from this group of mice were almost identical with the series shown in Pls. 1 and 2, figs. 1–6, except that the reversion to bacillary form occurred slightly sooner.)

Mice killed 14–18 *days after challenge.* 0 *hr. preparations.* Some animals showed only the granular and amorphous stages of the L-cycle. Others showed only the small round red form. 24 *hr. preparations.* Development varied according to the stage in which the organisms were isolated.

It was observed that the rapidity and completeness of the transformation to the L-form varied with the degree of immunity in the mouse. Those mice that had received an adequate immunizing dose (2000×10^6 organisms/mouse) followed by a 10-day to 3-week rest period yielded pure L-cultures on the fourth to seventh day after challenge. The mice that had received the smallest immunizing doses (80 × 10^6 organisms/mouse) followed by a long rest period yielded a mixture of L- and bacillary forms at this time as did the normal mice. However, 14–18 days after challenge even these mice yielded pure L-cultures. The most striking difference between immune and normal animals was the rapidity of phagocytosis, the immune ones showing more complete phagocytosis in 2 days than the normal ones did in 7 days. Furthermore, the extent of transformation to the L-form appeared to vary directly with the extent of phagocytosis. From these experiments it appeared that immunity in the mouse promoted the L-transformation.

Intraperitoneal inoculation of normal mice with the bacillary form

The peritoneum of the mouse is not a site favourable for the establishment of infection with *H. pertussis.* Small doses of the organism have little effect on the mouse while large doses produce rapid death from toxin. It was considered worthwhile, however, to investigate the behaviour of *H. pertussis* in this environment. Moderate doses of the bacillary form were injected into mice which were sacrificed at various times from 2 hr. to 7 days later. Cultures of the peritoneal fluid were made and impression preparations examined.

Mice killed 2 *hr. after inoculation.* 0 *hr. preparations.* About half the organisms were small round red forms or very pleomorphic pale blue forms in an intracellular position. Extracellular organisms were ordinary bacilli. 24 *hr. preparations.* Frequent small L-bodies as well as the small round red form were present intracellularly. Very numerous ordinary bacilli and a few small round red forms were present extracellularly.

Mice killed 1 *day after inoculation.* 0 *hr. preparations.* Nearly all organisms found were intracellular and in the small round red form. 24 *hr. preparations.* Small red form still present intracellularly, but many thin long bacilli were also present extracellularly.

Mice killed 6–7 *days after inoculation.* 0 *hr. preparations.* Minute round red forms in an intracellular position. No bacilli observed. 24 *hr. preparations.* Growth very slow. Size of red forms had increased slightly. Moderate number of pale blue, round or pleomorphic forms and a very few bacilli.

In normal mice phagocytosis was more prominent and rapid in the peritoneum than in the lung. As noted before, there appeared to be some correlation between phagocytosis and rapidity of transformation to the L-form. Therefore, although much the same sequence of events occurred in the peritoneum as in the lung, the transformation to the L-form was accelerated in the peritoneum.

Intraperitoneal inoculation of immunized mice with the bacillary form

The experiment was repeated using immunized mice. Mice were immunized with living or formalized bacilli with doses ranging from 2000 × 10^6 to 100 × 10^6. The rest period before challenge was 4–5 weeks.

Mice killed 2 *hr. after challenge.* 0 *hr. preparations.* Nearly all the organisms observed were intracellular and in the small round red form or very pleomorphic pale blue forms. 24 *hr. preparations.* Numerous large L-bodies and great numbers of small red forms both intracellular and extracellular. Many ordinary bacilli as well.

Mice killed 1 *day after challenge.* 0 *hr. preparations.* Nearly all organisms were intracellular and in some stage of the L-cycle, small round red forms, red granular forms, filaments, and red and blue L-bodies. 24 *hr. preparations.* Increased number of all the various L-forms. Numerous fat pale blue bacilli also present.

Mice killed 7 *days after challenge.* 0 *hr. and* 24 *hr. preparations.* No organisms at all could be found.

In the immune animal 1 day after challenge, the results in the peritoneum were much the same as in the lung. However, after a week not even the L-form could be found in the peritoneum. Possibly the conditions in the immune peritoneum are too unfavourable for any form of *H. pertussis* to survive for long.

DISCUSSION

It has been suggested elsewhere (Wittler, 1951) that the small round red form is a resistant form of *H. pertussis. In vitro* experiments showed that chilling, ageing, and the use of various chemicals promoted the transformation to L. The removal of these unfavourable environmental conditions resulted in the reversion from L to bacillary form.

The methods employed in the present studies have made possible a clear demonstration of the morphology of *H. pertussis* as it occurs *in vivo*. These studies show that the L-form of *H. pertussis* appears regularly in the mouse following intranasal and intraperitoneal infection. Furthermore, the L-form appears to be pathogenic, because most of the mice that received intranasal inoculation were ill or moribund when killed. Thus it seems unlikely that the small round red form is a degenerate type. The predominance of the L-form in immunized mice suggests that factors involved in host resistance may stimulate the transformation to L and that this transformation may assist the organism to escape destruction.

Work is now in progress on the virulence, toxicity, protective capacity, and many other aspects of the L-form. It is hoped that these experiments will aid in the interpretation of the significance of the transformation.

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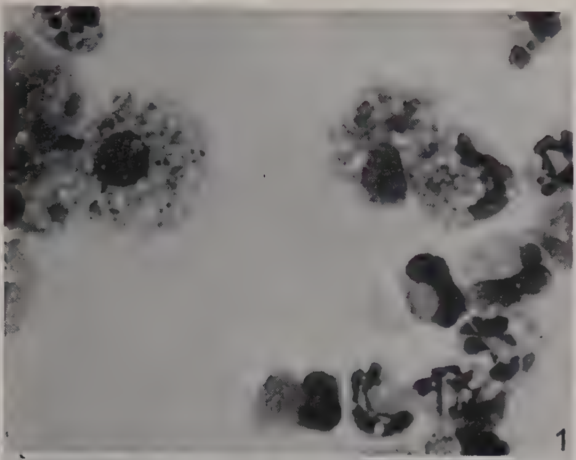

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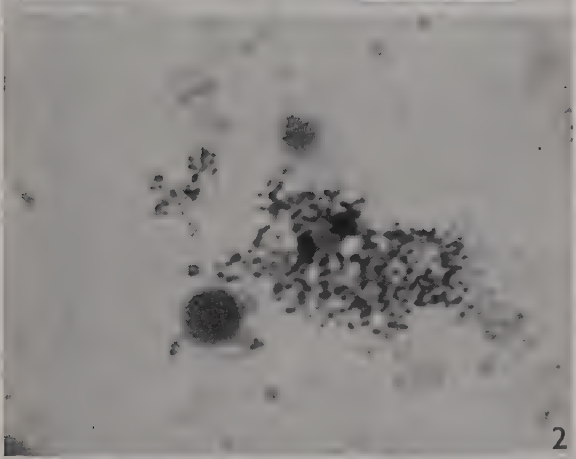

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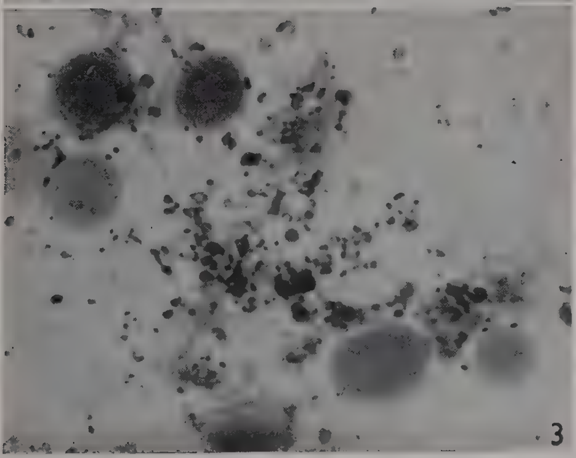

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R. G. Wittler—The L-form of *H. pertussis* in the mouse. Plate 1

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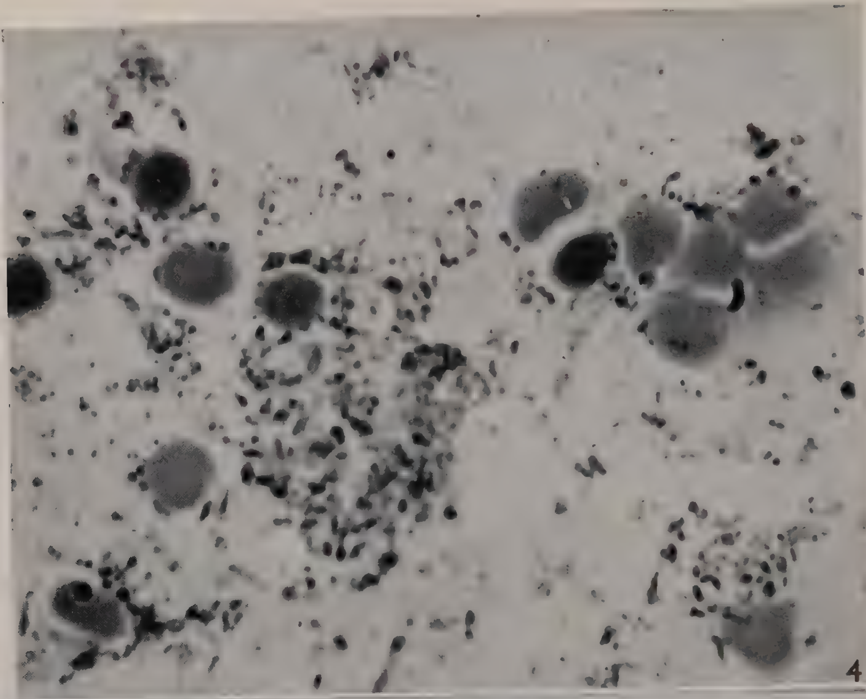

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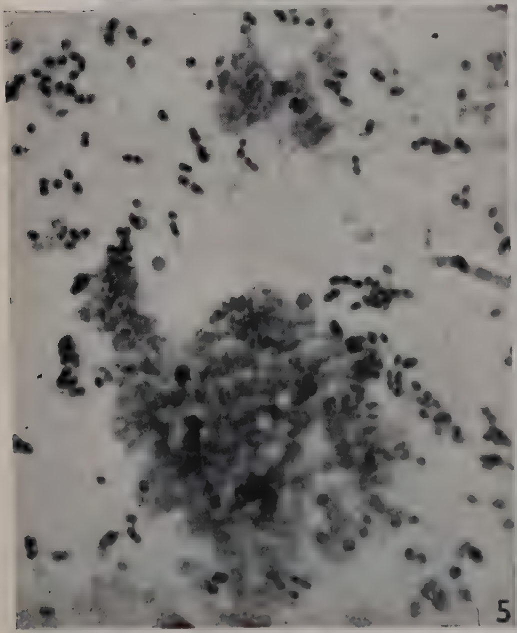

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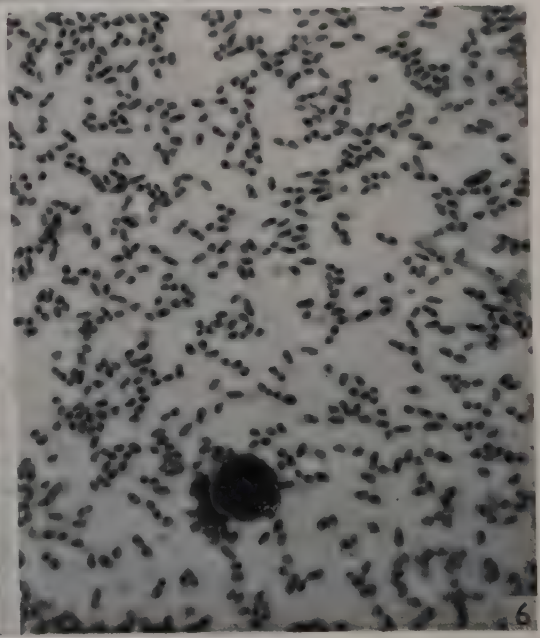

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R. G. Wittler—The L-form of *H. pertussis* in the mouse. Plate 2

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EXPLANATION OF PLATES

H. pertussis, strain 19939, isolated from a normal mouse inoculated intranasally with the L-form and killed 14 days later. Osmium tetroxide Giemsa impression preparations from BG plate cultures incubated at 35°. Figs. 1–4 treated for 3 min. at 55° with N-HCl. Figs. 5 and 6 untreated.

(Magnification ×2700 throughout.)

Plate 1

Fig. 1. 0 hr. preparation. Minute granular L-forms in the cytoplasm of leucocytes from the mouse lung.

Fig. 2. 3 hr. preparation. Small colony of L-forms next to an erythrocyte.

Fig. 3. 24 hr. preparation. Granular and amorphous L-forms. Several large bodies have appeared. Ring-shaped nuclear structure is characteristic of this stage of the L-cycle.

Plate 2

Fig. 4. 48 hr. preparation. A mass of amorphous L-material containing large and small bodies, granules, and a very few newly formed bacilli.

Fig. 5. 48 hr. preparation of first subculture. Large fat bacilli forming among masses of amorphous L-material.

Fig. 6. 24 hr. preparation of second subculture. Organisms have almost completely reverted to bacillary form.

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Moraxella: Classification and Taxonomy

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SUMMARY: Eleven strains regarded as moraxellas were studied; nine were characterized by moderate growth on the usual media, a positive oxidase reaction and sensitivity to penicillin, and six of these preferred or required a humid atmosphere when incubated at 37°. The classification of these strains was considered correct. Two strains did not show any of these characteristics, and it was concluded that they should not properly be classified as moraxellas.

A positive oxidase reaction, sensitivity to penicillin, and preference for a humid atmosphere at 37° may be useful criteria in the classification of *Moraxella*. It is suggested that *Moraxella* may be closely related to *Neisseria*, and that the description of the genus and its taxonomic position should be revised in accordance with Lwoff's proposals.

Mima polymorpha var. *oxidans* (De Bord) is believed to belong to *Moraxella*, whereas other members of the tribe Mimeae (De Bord) and *Bacterium anitratum* (Schaub & Hauber) are probably not closely related to *Moraxella*.

Lwoff (1939) proposed that the organism classified as *Haemophilus duplex* and some related organisms should be gathered in a new genus, *Moraxella*, and that this genus should be separated from the Haemophileae, as its species neither resembled the Haemophileae morphologically nor required haematin or phosphopyridinenucleotide as growth factors. These proposals were approved by Audureau (1940), who added a new species, *M. lwoffi*.

Bergey's Manual (1948) accepted Lwoff's proposal to create the genus *Moraxella*, but put it in the Haemophileae. The vague description of the genus—mainly morphological, which might fit almost any Gram-negative rod—indicates a lack of specific characters to differentiate it from other genera.

The present study was made to evaluate additional characters, which, added to the description, would clarify the taxonomy of the genus and show its relationship to organisms such as Mimeae (De Bord, 1939), *Bacterium anitratum* (Schaub & Hauber, 1948) and *Neisseria*, all of which appeared to resemble *Moraxella* in some respects.

MATERIAL AND METHODS

Of the strains isolated in this laboratory, one (3452/51) was from sputum (Henriksen, 1951), three from urine (283/49, 6758/51, 7146/51) and two (possibly the same strain) from throat cultures of a husband and wife (OT/51, AT/51). Type strains of some *Moraxella* species were received from the Institut Pasteur, Paris, through the courtesy of Dr A. Lwoff and Dr M. Piéchaud (*M. lacunata* 4238, Diplobacillus of Morax 260, Diplobacillus of Petit, *M. lwoffi* var. *bacteroides* and *M. pericardite*).

For comparison five strains of *Neisseria pharyngis*, four of the pigmented, carbohydrate-fermenting variety, and one non-pigmented, non-fermenting (*N. catarrhalis*), were studied (OT 2/51, OTJ 1/51, OTJ 2/51, TT/51, 26051/51).

Customary methods were used in the study of morphological and biochemical properties of the strains. For the study of sensitivity to antibiotics, three different methods were used.

(1) A filter-paper disk method (Jensen & Kiaer, 1948). Antibiotic solutions used to moisten the disks were: penicillin 175 units/ml., streptomycin 2800 μg./ml., sulphathiazole 6·6 mg./ml., chloromycetin and aureomycin 1000 μg./ml. each. Incubation at 28° for 48 hr.

(2) An antibiotic tablet method (Lund, Funder-Schmidt, Christensen & Dupont, 1951). The tablets (Roskilde Medical Co. Ltd., Roskilde, Denmark), which contain the above antibiotics and also terramycin, are deposited on peptone-free 10 % horse blood agar plates, which have been inoculated with a suspension of the organism to be tested. Inhibition zones are measured after incubation for 24 hr. at 37° in a closed jar containing some water.

(3) Titration in 10 % (v/v) serum broth containing twofold dilutions of the antibiotics. Incubation at 28° for 48 hr. Readings were made after 24 and 48 hr.

RESULTS

Morphology

The following characteristics (illustrated in Pl. 1, figs. 1–9) were observed in varying degree:

A preference for arrangement in pairs. In some strains nearly all organisms were arranged in pairs, as diplococci (283/49, fig. 1) or as short diplobacilli (3452/51, fig. 2 and a dissociant strain of 283/49); in others both diplo-forms and single cells were found; short chains were seen occasionally.

Plumpness of the cells. With the exception of two (*M. lwoffi* var. *bacteroides* and *M. pericardite*) all strains had plump, broad cells, usually with obtuse or nearly square-cut ends. The breadth of the cells varied between 1 and 1·5 μ., although larger cells were frequent in some strains. The length varied from neisseria-like coccus-forms to long filaments. The two exceptions had small coccoid or coccobacillary cells.

Pleomorphism. Considerable variations in size and shape were frequent, although not equally marked in all strains, nor under all conditions of growth. Large swollen cells could be found in all strains: there were spherical or oval bodies, club-shaped or sausage-shaped cells or coarse filaments with fusiform swellings. The strains *M. lwoffi* var. *bacteroides* and *M. pericardite* were less pleomorphic than most other strains.

Irregular staining. The majority of the cells were Gram-negative, but some cells with very sharp outlines were strongly stained by the counter-stain, whereas others were pale. Other cells, particularly the swollen elements, showed a tendency to be Gram-positive and were purplish to violet, or contained bluish black granules after decolorization with ethanol. Thorough treatment with ethanol removed all the purple stain. On the other hand, some of the swollen cells appeared as pale 'ghost cells'.

The individual strains showed considerable variations. One (283/49) produced diplococci nearly exclusively (figs. 1 and 6) and showed a striking resemblance to *N. gonorrhoeae*; only occasional rods could be found. A dissociant, split off by this strain in the course of the work, and also strain

3452/51 almost exclusively produced short plump, nearly square, diplobacilli (fig. 2). Strains 6768/51 (figs. 5, 9), 7146/51, OT/51 (fig. 4) and AT/51 produced mixtures of diplobacilli, diplococci and single cells, with rods predominating, whereas *M. pericardite* and *M. lwoffi* var. *bacteroides* generally produced very small coccoid and coccobacillary cells, frequently in pairs (fig. 3). Finally, strains *M. lacunata* 4238, Diplobacillus of Morax 260 (fig. 8) and Diplobacillus of Petit appeared to be in the R-form and grew as chains of elongated cells or as filaments.

Growth characteristics

The strains *M. lwoffi* var. *bacteroides* and *M. pericardite* grew vigorously on all media. The colonies were large and butyrous, resembling those of the Enterobacteriaceae, e.g. *Escherichia coli*. The strain *M. lwoffi* var. *bacteroides* produced slightly spreading colonies. Cultures of *M. pericardite* were characterized by a marked odour, resembling that of certain pseudomonas cultures.

The other strains showed a much less vigorous and slower growth. The colonies were smaller (*c.* 1 mm. or less after 24 hr.), grey or yellowish, raised, and friable or slightly viscous, like the colonies of some strains of *N. pharyngis*. A dissociant of 283/49 mentioned above, produced tiny, delicate, colourless, translucent colonies, resembling gonococcus colonies. One strain (3452/51) grew with mucoid colonies, resembling those of *Klebsiella ozaenae*. Finally the strains *M. lacunata* 4238, Diplobacillus of Morax 260, and Diplobacillus of Petit grew with very small, delicate, greyish colonies of a distinctly rough appearance.

Effect of temperature and humidity

Some of the strains (*M. lacunata* 4238, Diplobacillus of Morax 260, Diplobacillus of Petit, 283/49, 3452/51 and 6768/51) grew very poorly or not at all on blood agar plates incubated in an ordinary incubator at 37°. Growth was greatly improved by incubation in a closed jar containing some water. Growth at 28° was good regardless of the humidity and all strains showed growth at a room temperature of about 20°, although in some cases this was extremely poor and slow. Thus this group of strains was very sensitive to a dry atmosphere at 37°.

Strains 7146/51, OT/51, AT/51 and *M. pericardite* grew well under all these conditions, but *M. lwoffi* var. *bacteroides* did not grow at 37°, regardless of the degree of humidity; it grew vigorously at lower temperatures, including room temperature.

Growth requirements

Growth was studied on simple and enriched media and in the defined medium containing ethanol, citrate, ammonia and inorganic salts described by Audureau (1940). The strains could be divided in several groups according to growth requirements:

(1) Grow only on media containing body fluids: *M. lacunata* 4238, Diplobacillus of Morax 260.

(2) Grow on simple meat-infusion + peptone media but not in the defined medium: Diplobacillus of Petit, 3452/51, OT/51, AT/51.

(3) Grow on simple media; very slight but continuous growth (five transfers) in the defined medium, perceptible only as a very slight precipitate: 283/49.

(4) Moderate, slow growth in the defined medium: 6768/51, 7146/51.

(5) Vigorous, rapid growth in the defined medium: *M. pericardite*, *M. lwoffi* var. *bacteroides*.

Some biochemical reactions

The strains *M. lacunata* 4238, Diplobacillus of Morax 260, and Diplobacillus of Petit liquefied coagulated serum and, to some extent, coagulated egg; the others did not. Otherwise the biochemical reactions were the same as previously reported (Lwoff, 1939; Audureau, 1940; Henriksen, 1947; Piéchaud, Piéchaud & Second, 1951). None of the strains isolated in this laboratory fermented any of the usual carbohydrates.

All strains except *M. pericardite* and *M. lwoffi* var. *bacteroides* gave a positive oxidase reaction with tetramethyl-*p*-phenylenediamine. In some cases the reaction was as strong and rapid as in *N. gonorrhoeae*, in others it was weaker and slower. Young delicate colonies as a rule gave stronger and more rapid reactions than older and larger ones, possibly because the latter were not as wettable or as easily penetrated by the reagent.

Sensitivity to antibiotics

The results of the tests are shown in Table 1. The strains *M. lacunata* 4238, Diplobacillus of Morax 260 and Diplobacillus of Petit, due to poor and slow growth, did not give very sharp end-points in the serial dilution tests, nor very sharp zones in the other tests. In these cases the inhibition zones were recorded as averages of several measurements, and the results were checked after additional incubation, which brought out the limits of the zones better, apparently without appreciably changing their sizes.

Table 1. *Sensitivity of* Moraxella *and* Neisseria *strains to antibiotics*

	Penicillin			Streptomycin		Sulphathiazole		Chloromycetin			Aureomycin			Terramycin
Strains	I	II	III	I	II	I	II	I	II	III	I	II	III	II
M. lacunata 4238	58	50	0·05	55	40	60	19	—	38	0·8	—	28	0·4	35
Diplobacillus of Morax 260	54	50	0·025	45	35	0	(20)	42	36	0·8	42	27	0·8	30
Diplobacillus of Petit	60	45	0·025	50	35	30	21	55	35	0·8	53	28	0·4	28
283/49	38	30	0·025	35	30	0	0	46	32	6·3	37	25	1·6	28
3452/51	50	32	0·0125	41	39	12	13	48	38	6·3	50	27	0·8	31
6768/51	37	26	0·125	60	32	0	0	43	33	>12·5	35	16	0·4	26
7146/51	32	20	0·25	—	21	0	0	42	32	1·6	40	18	>12·5	28
M. lwoffi	(13)	—	>100	33	—	40	—	20	—	6·3	31	—	0·8	—
M. pericardite	0	0	>100	0	0	(28)	(20)	—	21	>12·5	24	18	3·1	21
OT/51	—	44	0·016	—	36	—	41	—	45	—	—	29	—	34
AT/51	—	52	0·016	—	43	—	49	—	52	—	—	38	—	40
N. pharyngis OT2/51	—	46	0·25	—	53	—	47	—	53	—	—	34	—	47
N. pharyngis OTJ1/51	—	45	0·125	—	52	—	49	—	54	—	—	33	—	46
N. pharyngis OTJ2/51	—	31	0·5	—	39	—	40	—	45	—	—	29	—	36
N. pharyngis TT/51	—	50	0·06	—	50	—	48	—	56	—	—	30	—	—
N. pharyngis 26051/51	—	35	0·5	—	41	—	41	—	47	—	—	31	—	37
Staph. aureus 1565	45	43	0·025	35	40	32	34	37	39	3·2	43	25	0·1	27

Column I: filter-paper disk method, diameter of inhibition zones in mm.
Column II: antibiotic tablet method, diameter of inhibition zones in mm.
Column III: titration in 10% serum broth. Lowest inhibitory concentration in μg./ml. (penicillin: units/ml.).
Figures in brackets indicate zones of only partial inhibition.

The most interesting results are those obtained with penicillin. These separate the strains into two groups; *M. pericardite* and *M. lwoffi* var. *bacteroides*, which were highly resistant to penicillin, in one group, and the other strains with great or moderate sensitivity, in the second. All strains were inhibited by concentrations within, or very close to, the usual therapeutic range, and all strains in the second group showed penicillin sensitivities within the range shown by various species of *Neisseria*.

With the strains of *N. pharyngis* there was a discrepancy between the results obtained by titration in a fluid medium and by the antibiotic tablet method. By the tablet method the strains appeared to be very sensitive to penicillin, but by the dilution method the sensitivity appeared to be only moderate. The reason for this discrepancy was not studied.

In my experience the antibiotic tablet method has shown some peculiarities, possibly due to different solubility and diffusion rates of the different antibiotics. The results obtained with penicillin, streptomycin, chloromycetin and sulphathiazole have usually been in good agreement with those obtained by other methods, but the inhibition zones obtained with terramycin and particularly with aureomycin have frequently been narrower than expected. This method should probably be considered to yield rough approximations rather than exact quantitative data.

DISCUSSION

The strains can be separated into two groups. Those of the first group are characterized by moderate growth on the usual media, colonies resembling species of *Neisseria*, a positive oxidase reaction, and strong to moderate sensitivity to penicillin. Several strains showed a marked preference for a humid atmosphere when cultivated at 37°. The individual cells are plump and broad and in some cases show a marked resemblance to *Neisseria* spp.

The second group, apart from some morphological and biochemical similarities, did not present these characteristics. It is doubtful whether these two groups should be classified in the same genus.

It seems reasonable to consider the oxidase-positive group, which agrees with the representatives of the type species, as moraxella strains, whereas the classification of the two remaining strains is an open question.

The description of the genus *Moraxella* given in *Bergey's Manual*, 1948, p. 590, does not allow the recognition of these two groups as none of the criteria used in separating them occurs in the description. It is difficult to assess the biological significance of the proposed new criteria, namely, oxidase reaction, penicillin sensitivity and sensitivity to a dry atmosphere, but they may be expressions of fundamental biological properties which might profitably be used in classification.

The oxidase reaction is not a common reaction and, apart from *Neisseria* and *Moraxella*, none of the recognized bacterial genera is known to give consistently positive reactions. Only occasional, often ill-defined, species outside these genera have been reported to give the reaction. In *Moraxella* the reaction has been reported to be positive by several authors (Oeding, 1946; Henriksen, 1947; Steen & Berdal, 1950).

It is commonly recognized that penicillin sensitivity is unusual in Gram-negative organisms, most of which are strongly or moderately resistant. The only important exceptions are *N. gonorrhoeae* and *N. intracellularis.* Little is known about the sensitivity of other species of *Neisseria,* but it seems that the non-pathogenic species are the less sensitive (Florey *et al.* 1949). My few strains appeared to be very sensitive when tested on a solid medium and moderately sensitive in a fluid medium, and were more sensitive than the majority of Gram-negative organisms.

The following statement by Dubos (1949) apparently might apply equally well to *Moraxella*: 'Although gonococci and meningococci are susceptible to both penicillin and gramicidin, this exception is correlated with the fact that the pathogenic Gram-negative cocci occupy an intermediate position between the Gram-positive organisms and the Gram-negative bacilli.'

Strains of *M. duplex* var. *liquefaciens* (Lwoff) isolated by Oeding (1946) and by Steen & Berdal (1950) were sensitive to 0·01 and 0·03 unit penicillin/ml. respectively. On the other hand, Cashell (1944) reported strains of Petit's bacillus and Morax-Axenfeld's bacillus to be insensitive *in vitro,* although eye inflammations were cured by local penicillin treatment. Milner (1944) and Sorsby (1945) successfully treated eye infections caused by these organisms, with penicillin.

The inability to grow in a dry atmosphere at 37° does not appear to be an equally constant property, since three of nine strains were exceptions, and therefore it may not be very significant in classification. It is interesting, however, that this characteristic is a further point of resemblance to the pathogenic neisseria.

The number of strains studied is too small for far-reaching conclusions; additional strains may show a wider range of variation. If, in future work, the main points should be corroborated, the description of the genus *Moraxella* might perhaps be revised along the following lines:

'Plump short rods or cocci, frequently with considerable variation of the size of the cells and a tendency to produce large swollen "involution forms", predominantly occurring in pairs and occasionally short chains. Some strains show striking resemblance to neisseria. Non-motile. Gram-negative, but some cells may show a tendency to retain some of the purple stain. Aerobic. Some strains prefer or require a humid atmosphere when cultivated at 37°. Oxidase reaction positive. Most strains are strongly or moderately sensitive to penicillin. Parasitic.'

Such a description would keep the genus reasonably homogeneous and prevent inclusion of organisms that, apart from morphological similarity, have little relationship to the original species. Some of the strains studied by Piéchaud *et al.* (1951) would have to be excluded, since one of their strains, which is probably included in this study (*M. pericardite*), did not belong to the oxidase-positive group; others were strains of the species *Bacterium anitratum* or the B 5 W group of Stuart, Formal & McGann (1949).

It is suggested that the division of the genus might follow the proposals of Lwoff (1939), by which one would recognize the species *lacunata* and *duplex* (with the variety *nonliquefaciens*). It has been shown in this study that at least some strains of these species possess the additional characteristics referred to before. The position of the strains isolated from eye inflammations in cattle (*M. bovis* in *Bergey's Manual*, 1948) is not clear. There is little evidence to show whether these strains differ sufficiently from the other species to deserve recognition as a separate species. In contrast to the other species these strains are reported to be haemolytic.

As for the species *lwoffi*, proposed by Audureau (1940), the fact that the only strain of this species that was available for examination (*M. lwoffi* var. *bacteroides*) differed from the oxidase-positive group, makes it desirable to re-examine other strains of the species. Of the strains isolated in this laboratory, two (6768/51 and 7146/51) grew fairly well in Audureau's defined medium, and thus might be classified as *M. lwoffi*, if this species is considered valid. One strain showed only a trace of growth in this medium and three strains refused to grow. Thus it seems that there may be gradual transitions between positive and negative strains. In other respects these six strains appeared to be similar, and it seems questionable whether differences in this single characteristic would justify the establishment of separate species. The significance of this characteristic, as well as the validity of the species *lwoffi*, should be reconsidered after further studies have been carried out.

Not only the description of the genus but also its taxonomic position may need revision, as suggested by Lwoff (1939). If need for either the V or the X factor is considered essential for inclusion in the Haemophileae, *Moraxella* has no natural place in this tribe. Only one of the species requires media enriched with body fluids, but is independent of the V and X factors, and the other species grow on simple media and even on a defined medium containing only citrate, ethanol, ammonia and salts. Therefore there seems to be little reason to retain *Moraxella* in the Haemophileae. Furthermore, all strains were characterized by comparatively plump, broad cells, coarser than those found in the Parvobacteriaceae (when the involution forms produced by some of the latter are disregarded). There appears to be no other strong argument for the inclusion of *Moraxella* in this family.

Two courses of action might be followed. If the evidence for inclusion of *Moraxella* in some other family be found insufficient, the genus might be placed temporarily in the Bacteriaceae until its final place could be agreed upon. Or one might try to find another family to which *Moraxella* shows relationship; in this case Neisseriaceae might be the best choice, as the following comparison shows:

	Neisseria	*Moraxella*
Shape of cells	Coffee-bean shaped diplococci	Short diplobacilli or coffee-bean shaped diplococci
Size	Often plump, but with considerable variation in many strains. Large swollen cells occur	Plump with considerable variation. Large swollen cells frequent
Staining	Gram-negative. Often irregular with strongly stained and pale cells	Gram-negative. Often irregular with strongly stained and pale cells. Some cells tend to resist decolorization

	Neisseria	*Moraxella*
Colonies	In some species soft, colourless. In others friable or viscous, grey or greyish white or pigmented. Small or moderate size	In some strains colourless, soft, in others friable or viscous, grey to yellowish grey. Small or moderate size
Growth	Some species require body fluids, others grow in simple media. Some species prefer a humid atmosphere at 37°	One species requires body fluids, others grow on simple or defined media. Some strains prefer or require a humid atmosphere at 37°
Oxidase reaction	Positive	Positive
Penicillin sensitivity	Some species strongly sensitive, others moderately sensitive or slightly resistant	Strongly or moderately sensitive
Habitat	Parasites in the respiratory or genito-urinary tract	Parasites in the respiratory or genito-urinary tract
Pathogenicity	Some species cause inflammations on mucous membranes.	Some species cause inflammations on mucous membranes

It is not likely that these similarities are entirely the result of coincidence, although the significance of all the characters given in the list may not be equally high. The only significant difference between the two genera is that one exclusively consists of cocci, and the other mostly of rods. Some strains of *Moraxella*, however, show such close resemblance to *Neisseria* that great care has to be exercised to avoid mistakes. Objection might be raised to the inclusion of rod-shaped and coccus-shaped organisms in the same family, but there is precedence for such an arrangement in the Lactobacteriaceae. It seems that the relationship between *Moraxella* and *Neisseria* may perhaps be as close as that between the tribes Streptococceae and Lactobacilleae. Before this proposal can be accepted the results reported here will have to be corroborated and extended by others, but it is suggested that the relationship between the two genera should be kept in mind.

The relationship of *Moraxella* to certain other organisms remains to be discussed. De Bord (1939, 1942, 1943, 1948) proposed a new tribe, Mimeae, composed of organisms showing morphological resemblance to neisseria. They were further characterized by pleomorphism, a tendency to retain the Gram stain and encapsulation. The genus *Mima* does not ferment carbohydrates, *Herellea* produces acid, and *Colloides* acid and gas from various sugars. Apart from these biochemical differences the three genera and their species (*Mima polymorpha* with the variety *oxidans*, *Herellea vaginicola* and *Colloides anoxydana*) are described with the same words, indicating that the other characters are identical. An analysis of these descriptions suggests the following considerations:

(i) The oxidase-positive variety of *Mima polymorpha* may be indistinguishable from *Moraxella duplex* var. *nonliquefaciens* (Lwoff, 1939). If so, the name suggested by De Bord would be invalid, as the relationship of this organism to the Morax-Axenfeld group had been recognized long before De Bord's paper appeared, even though De Bord's and Lwoff's papers both appeared in the same year.

(ii) The description of *Mima polymorpha* is the same as that of the variety *oxidans*, apart from the negative oxidase reaction. It may be suggested that more differences might have been found if the characters reported in this paper had been studied. It is impossible at present to decide where *M. polymorpha* belongs, and further studies are necessary. It is suggested that this species should not be included in the genus *Moraxella* until its properties have been re-examined.

(iii) *Herellea* shows considerable resemblance to *Bacterium anitratum* Schaub & Hauber (1948) or the B 5 W group described by Stuart, Formal & McGann (1949) and by Ferguson & Roberts (1950). Some strains isolated by Deacon (1945) and believed to be *Herellea* species, showed even greater resemblance to these organisms, as they fermented the same carboyhdrates, whereas De Bord's strains showed different fermentation reactions. This led Ewing (1949) to suggest a relationship between Mimeae, *Herellea* in particular, and *Bacterium anitratum*. Apart from morphological similarity there is no evidence to show that these organisms should be included in *Moraxella* as defined in this paper. *Bact. anitratum* is known to be resistant to penicillin (Schaub & Hauber, 1948) and has not been reported to be oxidase-positive. Piéchaud *et al.* (1951) have expressed a different opinion, as they consider the B5W group belongs to *M. lwoffi*.

(iv) *Colloides* gives exactly the same biochemical reactions as *Esch. freundii* (*Bergey's Manual*, 1948), and the unusual morphology appears to be the only reason for separating it from the latter species. Diplococcus-like cells in strains of various Enterobacteriaceae are not uncommon, and the grounds for establishing the genus *Colloides* may, therefore, be weak.

It is concluded that *Mima polymorpha* var. *oxidans* may belong to *Moraxella*, but the relationship of the other organisms mentioned will have to be decided when better evidence becomes available. It is felt that whereas morphological characters are very useful in bacterial classification in distinguishing between the larger taxonomic groups, over-emphasis on morphological details may lead to erroneous conclusions. This is particularly true of morphological characters which may be found more or less frequently in a number of widely separated taxonomic groups.

I am indebted to Dr A. Lwoff and Dr M. Piéchaud for strains of *Moraxella* and for friendly advice.

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EXPLANATION OF PLATE

Figs. 1–5 are living cells examined by phase contrast microscopy. Figs. 6–9 are cells stained by Gram's method with safranin as counter stain.

Fig. 1. *M. duplex* var. *nonliquefaciens*, strain 283/49. Nearly exclusively cocci, measuring *c.* 1·3–1·5 μ. × 1·3–1·7 μ. × 3600.

Fig. 2. *M. duplex* var. *nonliquefaciens*, strain 3452/51 (mucoid). Rods measuring *c.* 1·1–1·2 μ. × 2–3·5 μ. × 2800.

Fig. 3. *M. lwoffi* var. *bacteroides*. Cocci and short rods measuring *c.* 0·6–1 μ. × 0·8–2·2 μ. × 2500.

Fig. 4. *M. duplex* var. *nonliquefaciens*, strain OT 1/51. Short plump rods measuring *c.* 1·2–1·6 μ. × 1·4–2·6 μ. × 2500.

Fig. 5. *M. duplex* var. *nonliquefaciens*, strain 6768/51. Short diplobacilli measuring *c.* 1–1·1 μ. × 1·4–2·2 μ. × 2500.

Fig. 6. *M. duplex* var. *nonliquefaciens*, strain 283/49. × 1250.

Fig. 7. *Neisseria catarrhalis*, strain TT/51. Note striking similarity to fig. 6. × 1250.

Fig. 8. Diplobaccillus of Morax, strain 260. × 1250.

Fig. 9. *M. duplex* var. *nonliquefaciens*, strain 676851. × 1250.

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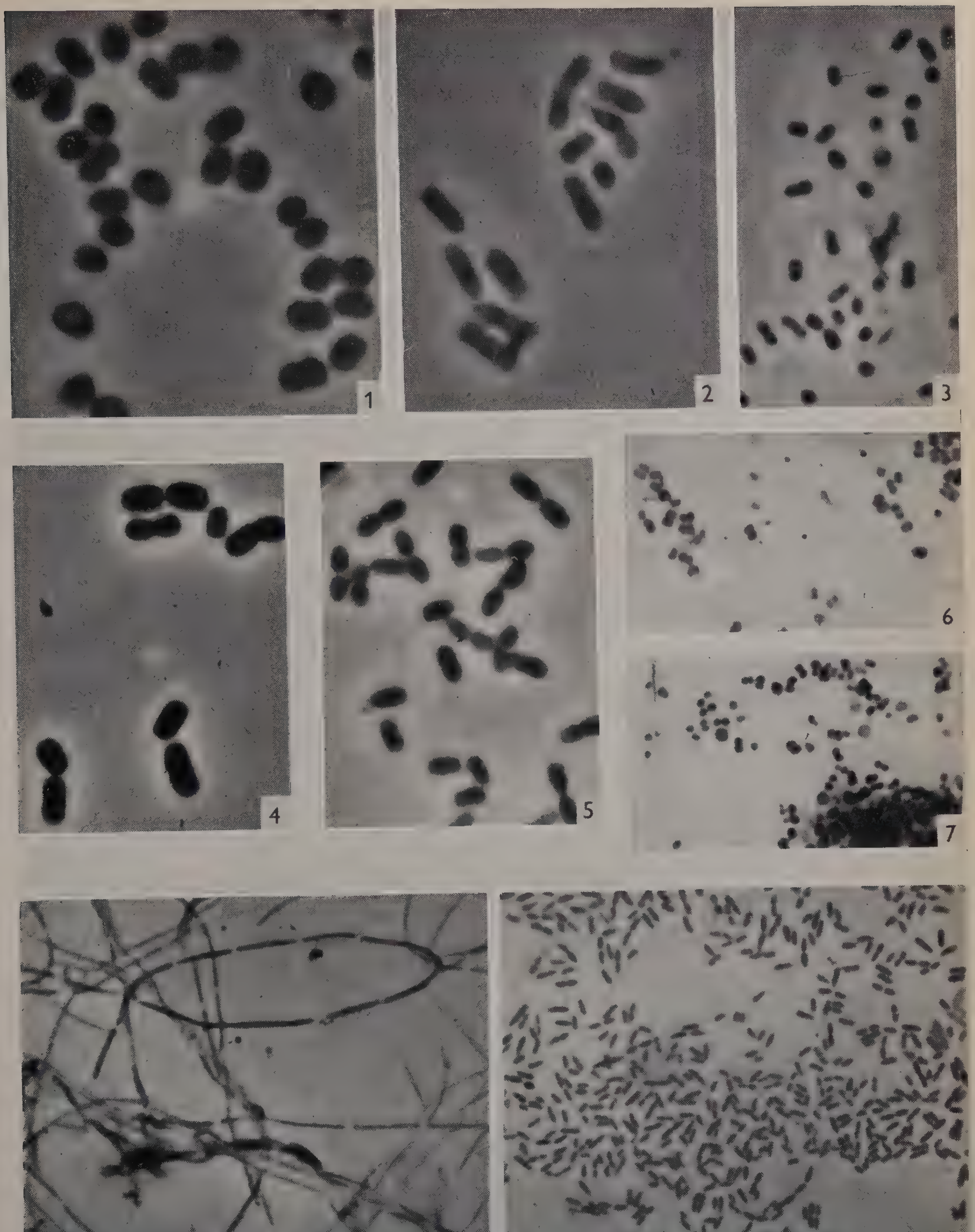

S. D. Henriksen—*Moraxella*: classification and taxonomy. Plate 1

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Uptake of Carbon Dioxide, Hydrogen and Oxygen by *Hydrogenomonas facilis*

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SUMMARY: The simultaneous uptake of CO_2, H_2, and O_2 by non-proliferating cells of *Hydrogenomonas facilis* was studied by means of a new method employing ordinary Warburg vessels each provided with one stopper side-arm and one vent side-arm. *H. facilis* effected the same overall reaction as that carried out by certain anaerobically adapted green algae: $6H_2 + 2O_2 + CO_2 \rightarrow (CH_2O) + 5H_2O$. In an atmosphere of CO_2 and H_2, no CO_2 fixation accompanied the quantitative reduction of nitrate to nitrite by molecular H_2; nor was there any change in concentration of bicarbonate or in total gas pressure when acetone, pyruvate, or α-ketoglutarate were added.

The interest attached to the comparative biochemistry of CO_2 fixation prompts the description of a technique for investigating the gaseous metabolism of *Hydrogenomonas facilis*, a bacterium which oxidizes molecular H_2 with O_2, and simultaneously assimilates CO_2 (Schatz & Bovell, 1952). The problem, then, is to measure the simultaneous uptake of CO_2, H_2 and O_2. To investigate the metabolism of certain anaerobically adapted green algae which fix CO_2 while carrying out the oxyhydrogen reaction, Gaffron (1942) employed specially constructed Warburg vessels. For the work with *H. facilis*, only the ordinary 2-side-arm (one stopper arm, one vent arm) flasks with centre wells were available. This made it necessary to develop the present method which allowed an analysis of the interrelationships among these three gases in the metabolism of *H. facilis*.

METHODS

The procedure for obtaining autotrophically grown cells of *H. facilis* for investigating its hydrogenase has already been described (Schatz & Bovell, 1952). Similar preparations were used to study fixation of CO_2. For this, the starting conditions were as follows:

Atmosphere: 10 % air (= 2 % O_2), 1 % CO_2, and 89 % H_2

Vent side-arm	Main vessel chamber	Stopper side-arm
Loosely rolled 1 × 2 cm. strip of filter-paper; 3–4 cm. of rubber tubing on end of vent	Cells suspended in 2·0 ml. M/30 Sørensen's phosphate buffer (pH 7·17); *c.* 85 μl. CO_2 present as dissolved bicarbonate	0·1 ml. freshly prepared aqueous pyrogallol plus 0·1 ml. 2N-H_2SO_4 containing 0·2 % $HgCl_2$

Volume of the flasks was 18 ml. (± 1 ml.). All experiments were carried out at 30°. Concentrations of O_2 and CO_2 much above those indicated proved impractical; when large residual volumes of unassimilated O_2 and CO_2 were

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absorbed (to be discussed) by the subsequently added alkaline pyrogallol, the Brodie solution was sucked into the main vessel chamber.

After the desired interval, the acid mixture from the stoppered side-arm was tipped in and instantaneously halted metabolism. Equilibration following the release of CO_2 from the bicarbonate usually required 15 min. To determine total CO_2 after tipping in acid, the open end of the manometer was corked and the assembly then removed from the water-bath and set in a rack without danger that the Brodie solution would be sucked over into the flask chilled to room temperature.

With a finely drawn Pasteur pipette, the vent arm channel and the rubber tubing were filled with 50 % KOH. A 0·2 ml. pipette charged with the same solution was firmly inserted in the rubber and the vent cautiously rotated to allow 0·200 ml. (or a similar accurately measured volume close to 0·200 ml.) of alkali to drain into the side-arm where it was immediately absorbed by the filter-paper. It was not necessary to replace all air in the vent arm channel and rubber joint, provided the alkali entered at a rate such that small trapped air bubbles neither rose into the pipette nor were driven down into the side-arm. After return of the assembly to the bath and removal of the cork from the end of the manometer, the absorption of CO_2 was complete in *c.* 15 min.

Mixing the acidified killed cell suspension in the main vessel chamber with the KOH-filter-paper in the vent side-arm produced alkaline pyrogallol which absorbed all residual O_2 within 25 min.

The determination of bicarbonate was direct. The values obtained for total CO_2 and O_2 were obtained indirectly and represented the unconsumed fractions of these gases. The amounts of CO_2 and O_2 actually assimilated by *H. facilis* were calculated by subtraction:

$$\text{total initial gas} - \text{unused or residual gas} = \text{gas consumed by cells.}$$

To determine the consumption of H_2, the CO_2, H_2 and O_2 initially present only as gases were considered. Since N_2 was not fixed by *H. facilis* (Schatz & Bovell, 1952) nor absorbed by alkaline pyrogallol, it was inert and disregarded in the computations. However, the increase in volume of liquid resulting from the addition of 0·200 ml. of KOH solution through the vent affected manometric readings as would a non-compressible fluid. For the most accurate work this necessitated the use of corrected values for K_{O_2} and K_{CO_2} (Umbreit, Burris & Stauffer, 1949) in all calculations following the introduction of alkali.

Since the solubilities of O_2 and H_2 are small and numerically close:

$$\alpha_{H_2}^{30^\circ} = 0{\cdot}02 \quad \text{and} \quad \alpha_{O_2}^{30^\circ} = 0{\cdot}03$$

the oxygen constant K_{O_2} was employed for both gases (as was done by Gaffron, 1942) as well as in the computations for the volume of alkali added through the vent.

In computing the amount of each gas assimilated ($-\Delta CO_2$, $-\Delta H_2$ and $-\Delta O_2$), the following designations were employed:

Time	Mm. manometer reading	Gas
Zero (start of experiment)	a	$C_b = CO_2$ present as bicarbonate. C_i, H_i and O_i = total initial CO_2, H_2 and O_2 present as gases
After tipping in acid	b	$C_b = CO_2$ released from bicarbonate in the control flask. C_f = total final (unused) CO_2
After addition of KOH to vent arm	c	O_f = total final (unused) O_2
After KOH tip	d	H_f = total final (unused) H_2

The bicarbonate content of the control flask was taken as a constant for all vessels since the pH, the volume of liquid, and the partial pressure of CO_2 were identical in all vessels at the beginning of the experiment. The atmospheric concentration in terms of μl. of CO_2, H_2 and O_2 in all reaction vessels at zero time was calculated by direct proportion from the values for V_g, the gas volume which was known for all flasks, and the atmospheric concentration of the control vessel, which was analysed at the beginning of the experiment. In the control vessel,

$$-\Delta CO_2 = -\Delta H_2 = -\Delta O_2 = 0,$$

so that

$$C_f = C_i + C_b, \quad H_f = H_i \quad \text{and} \quad O_f = O_i.$$

Thus, for computing $-\Delta CO_2$, let $200/K_{O_2}$ = mm. increase in manometric reading due to introduction of 200 μl. of KOH solution into the vent side-arm; and $b + 200/K_{O_2}$ = theoretical manometric reading if KOH absorbed no gas. But since C_f is quantitatively removed by KOH,

$$b + 200/K_{O_2} - C_f/K_{CO_2} = c,$$

from which

$$C_f = K_{CO_2}\,(b - c + 200/K_{O_2}).$$

Therefore,

$$-\Delta CO_2 = C_i + C_b - C_f = C_i + C_b - K_{CO_2}\,(b - c + 200/K_{O_2}).$$

In the control vessel,

$$-\Delta CO_2 = 0,$$

so that

$$C_i = K_{CO_2}\,(b - c + 200/K_{O_2}) - C_b.$$

This expresses the relationship between the concentration of bicarbonate and gaseous CO_2 at zero time.

After mixing the acidified cell suspension in the vessel with KOH on the filter-paper in the vent side-arm, both C_f and O_f are completely absorbed by the alkaline pyrogallol. As a result

$$c - O_f/K_{O_2} = d, \quad \text{or} \quad O_f = K_{O_2}\,(c - d),$$

and

$$-\Delta O_2 = O_i - O_f = O_i - K_{O_2}\,(c - d).$$

To calculate $-\Delta H_2$, let

$$H_i/K_{O_2} = a - C_i/K_{CO_2} - O_i/K_{O_2},$$

and

$$H_f/K_{O_2} = d - 200/K_{O_2}.$$

$$-\Delta H_2 = H_i - H_f = K_{O_2}\,(a - d - C_i/K_{CO_2}) - O_i + 200.$$

For simplicity and convenience in deriving the above relations, vessel-constant corrections due to the volume of KOH added through the vent have not been introduced. In experiments where close approximations are adequate, it is not necessary to apply the corrections for K_{O_2} and K_{CO_2}.

RESULTS

A typical experiment with 3-day old autotrophically grown cells of *H. facilis* is presented in Table 1. It is evident, disregarding the very low endogenous respiration, that the following reactions are effected by *H. facilis*:

$$
\begin{aligned}
4H_2 + 2O_2 &\longrightarrow 4H_2O, \\
2H_2 + CO_2 &\longrightarrow H_2O + (CH_2O) \\
\hline
6H_2 + 2O_2 + CO_2 &\rightarrow 5H_2O + (CH_2O)
\end{aligned}
$$

This is the same overall reaction carried out by Gaffron's anaerobically adapted green algae (1942).

Table 1. *Assimilation of* CO_2, H_2 *and* O_2 *by* Hydrogenomonas facilis

0·25 mg. dry weight equivalent of cells/vessel. Q values calculated from the 60 min. data.

		Time of observation (min.)				
	KOH in centre well*	20	40	60	80	100
Uptake of		µl. gas consumed				
CO_2 (as HCO_3^-)	−	11	26	35	48	51
CO_2 (total)	−	14	26	38	46	55
O_2	+	27	54	76	102	114
	−	31	56	78	91	118
H_2	+	63	116	166	200	240
	−	90	164	234	278	335
$H_2:O_2$	+	2·3	2·1	2·2	1·9	2·1
	−	2·9	2·9	3·0	3·1	2·9
$H_2:O_2:CO_2$	−	6·4:2·2:1·0	6·3:2·1:1·0	6·2:2·0:1·0	6·1:1·9:1·0	6·1:2·1:1·0
Q_{CO_2}	−	.	.	72	.	.
Q_{O_2}	+	.	.	145	.	.
	−	.	.	148	.	.
Q_{H_2}	+	.	.	315	.	.
	−	.	.	446	.	.
		Endogenous respiration				
O_2	+	0	4	6	8	9
Q_{O_2}	+	.	.	12	.	.

* KOH + : atmosphere = 10 % air + 90 % H_2. KOH − : atmosphere = 10 % air + 1 % CO_2 + 89 % H_2.

In other experiments under anaerobic conditions described in Tables 2 and 3, the fixation of CO_2 could not be demonstrated. During the reduction of nitrate to nitrite with molecular H_2, the ostensible fixation of 2 µl. of CO_2 lies well within the experimental error for Warburg work.

According to calculations from the values cited by Rabinowitch (1945) and Stephenson (1949), both the heat content and free energy from the reduction of nitrate to nitrite by molecular H_2 are approximately two-thirds of the corresponding values for the oxyhydrogen reaction with an equivalent amount of H_2. For this reason, it was anticipated that during the reduction

$$H_2 + 1/2\ O_2 \rightarrow H_2O + 69\ \text{kcal.},\quad \Delta F = -56\ \text{kcal.}$$

$$NO_3^-\ (\text{aq.}) + H_2 \rightarrow NO_2^- + H_2O + 45\ \text{kcal.},\quad \Delta F = -39\ \text{kcal.}$$

of nitrate about two-thirds as much CO_2 would be assimilated per mole of H_2 oxidized with nitrate as compared with O_2.

Table 2. *Inability of* Hydrogenomonas facilis *to fix* CO_2 *during the reduction of nitrate by molecular* H_2

6·0 mg. dry weight equivalent of cells/vessel. Q values calculated from the 50 min. interval required for the actual reduction of nitrate.

	Atmosphere			
	100 % H_2 KOH in centre well		1 % CO_2 + 99 % H_2 No KOH	
	µmole KNO_3 tipped from side-arm			
	0	10·0	0	10·0
Observed µl. gas consumed	0	217	0	225
µl. H_2 required for reduction of all NO_3^- to NO_2^-	0	224	0	224
Total µl. CO_2 present at time 0	.	.	295	299
Total µl. CO_2 present at end	.	.	293	297
µl. CO_2 fixed	.	.	2	2
Q_{H_2}	.	41	.	43
Q_{CO_2}	.	.	0·4	0·4
Endogenous fermentation				
Total µl. CO_2 evolved	.	.	−2·0	.
Q_{CO_2}	.	.	−0·4	.

The inability of *H. facilis* to fix CO_2 during the reduction of nitrate was surprising. The organism grows well autotrophically with nitrate as the sole source of nitrogen, and such cultures give an intense test for nitrite (Schatz & Bovell, 1952). This ruled out the possibility that nitrate inhibited the chemosynthetic assimilation of CO_2 by *H. facilis* as it does the photosynthetic fixation of CO_2 by *Chlorella pyrenoidosa* (Warburg & Negelein, 1920), which van Niel (1949) has considered to be a competition between nitrate and CO_2 as H-acceptors in the photolysis of H_2O. Since *H. facilis* failed to apply the energy released from the oxidation of H_2 by nitrate for the fixation of CO_2 anaerobically, it differs from *Thiobacillus thiooxidans*. Vogler & Umbreit (1942) found that *T. thiooxidans* was capable of accumulating in organic phosphate esters the energy derived from the aerobic oxidation of sulphur in the absence of CO_2, and subsequently utilizing this energy store for the assimilation of CO_2 under anaerobic conditions.

H. facilis also did not consume CO_2 in an atmosphere of H_2 and CO_2 even after 20 hr. (Table 3). With *Rhodospirillum rubrum*, the photoreduction of

CO_2 began only after an adaption period of 8 to 9 hr. (Foster, 1944). With *H. facilis* there was no uptake of CO_2 or H_2 in the presence of pyruvate, α-ketoglutarate, or acetone (Table 3). The assimilation of CO_2 is in considerable measure mediated by a carboxylation of pyruvate and α-ketoglutarate (Ochoa, 1951).

Table 3. *Inability of* Hydrogenomonas facilis *to assimilate* CO_2 *and* H_2 *in the presence of pyruvate, α-ketoglutarate, or acetone*

Supplement tipped from side-arm	Atmosphere	Duration of experiment (hr.)	Dry weight equivalent of cells (mg.)	Results
Pyruvate,* 10·0 μmole	2% CO_2+98% H_2	2·8	16	No change in bicarbonate concentration or manometric reading. Rapid gas uptake upon introduction of air
α-Ketoglutarate,* 10·5 μmole	1% CO_2+99% H_2	1·4	13	
Acetone, 10·0 μmole	1% CO_2+99% H_2	1·8	11	
None	1% CO_2+99% H_2	20	30	
None	1% CO_2+89% H_2+ 10% air	1	0·25	38 μl CO_2 fixed. (Table 1)

* Added as sodium salts.

DISCUSSION

The respirometric method of analysis developed here requires actually only the determination of bicarbonate, and total initial CO_2 and O_2 gases in the control vessel at zero time. With this information plus the values for the gas volume of the filled flask, the constants K_{O_2} and K_{CO_2}, and the manometric readings *a*, *b*, *c*, and *d* corrected for thermobarometric variation, it is easy to calculate the net exchange of H_2, O_2, and CO_2 independently in flasks gassed from the same mixture. Despite the numerous operations to which each manometer is subjected, the results obtained have been well within the range of experimental error conventionally accepted for simpler operations with the Warburg apparatus.

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Respiration of *Penicillium chrysogenum* in Penicillin Fermentations

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SUMMARY: Spore-inoculated penicillin fermentations using *Penicillium chrysogenum* W48–701 were carried out in two different types of equipment: stirred and aerated laboratory fermentors, or shaken flasks. The respiration of the mycelium was measured in Warburg manometers. The maximum oxygen demand in the fermentations occurred at approximately 40 hr. and the maximum Q_{O_2} of the mycelium at 24 hr. or earlier. During the whole phase of penicillin formation, respiration decreased steadily. Mycelium grown in the stirred and aerated vessels showed the highest respiration rate. In the shaken flasks the rate of solution of oxygen in the medium was found by polarographic methods to be insufficient to allow a respiration rate as high as that which occurred in the stirred and aerated vessels. Respiration in shaken flasks, however, was not directly limited by the rate of solution of oxygen but by the amount and kind of the respiratory enzymes which appear to be synthesized in proportion to the concentration of dissolved oxygen present during early growth.

Whereas the respiratory quotient (R.Q.) of mycelium from shaken-flask fermentations remained approximately unity throughout the fermentation, in the stirred and aerated vessels the R.Q. of the mycelium fell from unity to as low as 0·57, during the fermentation. The degree of aeration therefore appears to influence not only the quantity but also the kind of respiratory enzymes synthesized.

Studies on the oxygen requirements of *Penicillium chrysogenum* in submerged fermentations have been largely concerned with determining the optimum conditions of aeration for maximum penicillin production (Brown & Peterson, 1950; Bartholomew, Karow, Sfat & Wilhelm, 1950). Less attention has been paid to the respiratory activities of the mycelium, particularly with respect to different types of fermentation equipment. The experiments reported here were designed to determine the oxygen demands of mycelium throughout fermentations and the effect of different degrees of aeration on the respiration of the organism.

METHODS

Fermentation methods. Laboratory fermentors of the type described by Lumb & Fawcett (1951) containing 3 l. of medium were operated with an air flow of 2 vol./vol. medium/min. and a stirring speed of 1000 r.p.m. Shaken-flask fermentations were carried out with 75 ml. medium in 250 ml. conical Pyrex flasks on a rotary shaker having a speed of 220 r.p.m. and a throw of 1½ in. All fermentations were carried out at 24–26°.

The medium used in all the experiments contained: corn steep liquor, solids basis, 1·5 %; lactose, 3·0 %; glucose, 0·5 %; $NaNO_3$, 0·3 %; KH_2PO_4, 0·05 %; Na_2SO_4, 0·1 %; pH value adjusted to 5·8. Phenylacetic acid was added to the stirred and aerated fermentations in additions of 0·05 % every 12 hr. starting at the 24th hr. In the shaken-flask fermentations a concentration of 0·25 %

phenylacetic acid was usually included in the medium before autoclaving. While penicillin production was then very satisfactory, early growth in the shaken flasks appeared to be retarded by the initial presence of phenylacetic acid. Since the purpose of the present work was to compare the respiratory activities of mycelium rather than to produce maximum penicillin titres, phenylacetic acid was consequently omitted from all the shaken-flask fermentations described here. Lard oil containing 2·5 % cetyl alcohol was used as an antifoam agent when required. All fermentations were inoculated with a spore suspension of *Penicillium chrysogenum* W 48–701.

Manometric methods. Conventional Warburg techniques were used as described by Umbreit, Burris & Stauffer (1945).

Determination of the efficiency of aeration in the fermentation equipment. The polarographic methods described by Wise (1951) were used. Aeration is measured in terms of a constant, ϕ_L, which is dependent on the rate of air flow, agitation, shape of vessel, etc. The value of ϕ_L for different conditions of aeration was determined by the gassing-out method of Wise (1951). The value of ϕ_L characterizes the efficiency of aeration in a particular vessel for a given air flow and rate of agitation. The minimum value of ϕ_L necessary to ensure an adequate rate of solution of oxygen depends on the oxygen demand of the organism. It was shown by Wise (1951) that if the rate of solution of oxygen in a liquid is expressed by the equation

$$\frac{dC}{dt}=\phi_L(C_e-C),$$

where C is the concentration of dissolved oxygen at time t and C_e the concentration of oxygen when the solution is saturated with air, the maximum rate at which oxygen can dissolve is given by $\phi_L C_e$, since the rate of solution of oxygen will be maximum when $C=0$. Therefore if k_0 represents the oxygen demand of mycelium the fermentation will be adequately aerated if $\phi_L C_e > k_0$, hence the minimum value of ϕ_L necessary for adequate aeration can be obtained. The value of k_0 expressed in arbitrary polarograph units is obtained by plotting the uptake of dissolved oxygen in the polarograph cell against time. C_e can be obtained by filtering off the mycelium and saturating the sample with air. By these methods the actual value of ϕ_L in a particular fermentation can be obtained and also the minimum value of ϕ_L necessary in that fermentation to ensure an adequate supply of oxygen to the mycelium. It is thus possible to assess the state of aeration in a fermentation.

Mycelial weight. Dry mycelial weights were determined by filtering a measured volume of a fermentation sample on a tared filter-paper in a Buchner funnel, drying overnight at 80° and weighing.

RESULTS

Aeration conditions in the fermentation equipment

The values of ϕ_L (defined above) for the stirred and aerated vessels and shaken flasks are shown in Table 1. In all the fermentations in the stirred and aerated vessels stirring was at 1000 r.p.m. and air flow 2 vol./vol. medium/min.

It will be seen from Table 1 that the value of ϕ_L under these conditions was >10. At the time of peak oxygen demand (determined from preliminary manometric experiments) the minimum value of ϕ_L necessary to ensure an adequate rate of solution of oxygen to meet the demand of the mycelium was found to be 0·78 (average of three fermentations). The rate of solution of oxygen in such fermentations therefore was greatly in excess of the maximum demand. Even at the peak oxygen demand the fermentation medium was 80–90 % saturated with air.

It will be seen from Table 1, however, that in shaken-flask fermentations the value of ϕ_L was 0·43. The rate of solution of oxygen was therefore not sufficient to meet a demand as high as that shown by mycelium from the stirred and aerated fermentations, for which a minimum value of 0·78 was required. The oxygen uptake of mycelium in shaken flasks therefore must be lower than that of mycelium in the highly aerated vessels. From these experiments it is clear that the two types of fermentation equipment gave widely different conditions of aeration.

Table 1. *Values of ϕ_L in stirred and aerated vessels and in shaken flasks*

Stirred and aerated vessels

Stirring (r.p.m.)	Air flow (vol./vol./min.)	ϕ_L
1000	2	>10
740	2	7
730	1	3·2
420	1	1·12
350	1	0·46
300	1	0·3
850	0·2	1·4
550	0·2	0·65
440	0·2	0·48

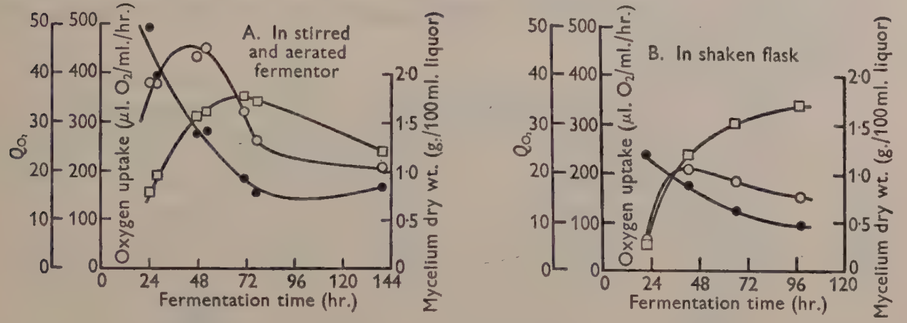
Shaken flasks

(r.p.m.)	Throw	ϕ_L
220	1·5 in.	0·43

Oxygen uptake in the stirred and aerated vessels

Samples were removed at intervals from the fermentation vessels and the uptake of oxygen measured in Warburg manometers at the temperature of the fermentation at that time. To ensure that the rate of solution of oxygen in the Warburg flasks was not a limiting factor, the rocking rate of the flasks was increased until no further increase in the rate of oxygen uptake occurred. With samples of 1 ml. fermentation liquor and 1 ml. sterile distilled water/flask it was found that a rocking rate of 120 strokes/min. provided an ample margin in the rate of solution of oxygen for all samples. A measured volume of the sample was used to determine mycelial dry weight.

The results of a typical experiment are shown in Fig. 1*A*. The oxygen demand of the fermentation rose sharply during the early stages and reached a maximum at 36–48 hr. After this, despite continued mycelial growth the oxygen demand decreased steadily and was at less than half the maximum rate when the accumulation of penicillin was at a maximum, at about 4 days. Penicillin production did not begin until *c.* 36 hr. and then occurred at a uniform rate until *c.* the 96th hr. Over almost the entire period of penicillin production the oxygen demand fell steadily, and during the later stages of penicillin formation the weight of mycelium also decreased. In these fermentations the production of penicillin was clearly not a direct function of mycelial growth or of oxygen uptake but was rather the result of independent reactions which may, however, be influenced by the earlier respiratory activities of the mycelium.



A *B*

Fig. 1. Oxygen uptake and growth of *Penicillium chrysogenum* W 48–701 in spore-inoculated penicillin fermentations. Oxygen uptake/ml. brew = ○—○—○; Q_{O_2} = ●—●—●; growth = □—□—□. *A*, in laboratory fermentor stirred at 1000 r.p.m. with air flow, 2 vol./vol. liquor/min.; *B*, in shaken flask.

The respiration rate of mycelium as distinct from the oxygen uptake/ml. fermentation liquor is expressed as $\mu l.O_2$ taken up/mg. dry mycelium/hr. (Q_{O_2}) in Fig. 1. It will be seen that the Q_{O_2} was highest at the earliest time of sampling, i.e. 24 hr. and continued to decrease steadily throughout the rest of the fermentation period. At the time of maximum penicillin production the rate of respiration was relatively low.

Oxygen uptake in shaken flasks

Shaken-flask fermentations were sampled at intervals and the oxygen uptake measured as in the experiments with the highly aerated fermentations. The results of a typical experiment are shown in Fig. 1*B*. Although the peak oxygen demand again occurred at 36–48 hr. the rate of oxygen uptake was very much lower than in the highly aerated vessels. It was evident that this would be so from the rate of solution of oxygen in the shaken flasks. In the latter the maximum rate of oxygen uptake was only half that of mycelium from the highly aerated fermentations. In addition, the rate of growth was slower and so was the rate of penicillin production.

The effect of aeration on the respiratory quotient

In the highly aerated fermentations the volume of carbon dioxide evolved during the first 36 hr. corresponded closely to the volume of oxygen taken up thus giving a respiratory quotient (R.Q.) of approximately unity. After 36 hr., however, the R.Q. fell and reached a value of 0·7–0·6 during the period of maximum rate of penicillin production. On occasion the R.Q. was as low as 0·57 after only 50 hr. In contrast with this effect, the evolution of carbon dioxide in the shaken-flask fermentations closely paralleled the oxygen uptake throughout the greater part of the fermentation. An R.Q. of approximately 1·0 was therefore maintained without the pronounced decrease which occurred in the highly aerated fermentations. The R.Q. values for two typical experiments are shown in Table 2.

Table 2. *The respiratory quotients of penicillin fermentations in shaken flasks and stirred and aerated vessels*

Shaken flasks		Stirred and aerated vessels	
Hr.	R.Q.	Hr.	R.Q.
21	1·27		
26	1·12	24	1·0
43	1·05	46	0·87
50	1·02	51	0·80
66	0·94	69	0·72
74	1·05		
98	0·81	94	0·60

The continued high R.Q. value in shaken flasks and the falling values in the highly aerated fermentations were shown in all the experiments carried out and appeared to represent some fundamental difference in the respiratory activities in the two types of fermentation.

The effect of aeration on the synthesis of respiratory enzymes

It will be seen from Fig. 1*A* & *B* that the rate of respiration (Q_{O_2}) of mycelium grown in shaken flasks was very much lower than that of mycelium from the highly aerated fermentations. The average of the maximum Q_{O_2} values of three such shaken-flask fermentations was 37·0, whereas that of three parallel fermentations in the stirred and aerated vessels was 71·7. All the measurements of the respiration rates, however, were carried out under conditions which ensured an excess of available oxygen. Despite this excess of oxygen in the Warburg manometers the mycelium obtained from fermentations in the two types of equipment showed this marked difference in respiration rate.

It would appear therefore that a less active respiratory mechanism is elaborated in the shaken flasks compared with the highly aerated fermentations, and that it is the activity of this mechanism that limits respiration, not the immediate supply of available oxygen. Since a more active respiratory mechanism is synthesized under the conditions of the higher degree of aeration it would appear that the concentration of dissolved oxygen during the early period of growth influences the synthesis of respiratory enzymes. Not only the quantity but also the kind of respiratory enzymes synthesized appears to be influenced by the degree of aeration. Attention has already been drawn to the results given in Table 2 which show that different types of metabolism take place in the two different sets of fermentation equipment. A continued high R.Q. was shown by mycelium from the shaken flasks and a lower and decreasing R.Q. by mycelium from the highly aerated fermentations. This difference in metabolism although apparently resulting in the first place from the difference in aeration is little influenced by the immediate concentration of available oxygen. The respiration of the two types of mycelium under the reverse conditions is shown in Table 3. Mycelium grown under conditions in the high degree of aeration present in the stirred vessels showed the same type of respiration, as indicated by the R.Q., when the oxygen supply was limited in the Warburg flasks by not rocking them as when an excess of dissolved oxygen was provided by rocking the flasks at 150 strokes/min. Conversely the typical R.Q. value of *c.* unity shown by shaken-flask mycelium was also as apparent when an excess of oxygen was provided as when the supply was limited.

Table 3. *The effect of different conditions of available oxygen on the respiration of mycelium from shaken flasks and from stirred and aerated vessels*

Age of mycelium (hr.)	Manometer rocking rate (strokes/min.)	Mycelium from shaken flasks			Mycelium from stirred and aerated vessels		
		O_2 taken up (µl./ml./hr.)	CO_2 evolved (µl./ml./hr.)	R.Q.	O_2 taken up (µl./ml./hr.)	CO_2 evolved (µl./ml./hr.)	R.Q.
46	150	273	264	0·97	426	246	0·58
46	0	140	144	1·03	176	128	0·73
50	150	247	227	0·92	421	239	0·57
50	0	98·7	95	0·96	134	73	0·54

The difference in metabolism is therefore not due to the selective functioning of different enzyme systems in response to the immediate supply of oxygen. The degree of aeration in the fermentation appears rather to influence the synthesis of different enzymes so that different enzymic equipments exist in the two types of mycelium.

DISCUSSION

These experiments show that the oxygen demand does vary considerably as the fermentation proceeds and is greatest at a relatively early stage. The time of peak oxygen demand would probably be even earlier if a vegetative inoculum were used. In shaken-flask fermentations the maximum oxygen demand occurred at approximately the same time as in the highly aerated fermentations, but was, however, considerably lower. In shaken flasks the rates of growth and of penicillin production were slower than in the stirred and aerated vessels.

Although a high level of aeration is necessary for maximum penicillin yields, the peak oxygen demand does not coincide with the time of maximum penicillin formation. During penicillin formation, which occurs at an almost uniform rate, the respiration rate decreases steadily. It would appear that high aeration is therefore not directly required for the process of penicillin formation, but rather for the formation of the enzyme mechanism necessary for penicillin production and that, once formed, this mechanism does not function in proportion to the subsequent rate of growth or rate of respiration.

The fact that mycelium when grown under conditions of low aeration and subsequently subjected to an increased supply of available oxygen continues to respire at a slower rate than mycelium grown initially under conditions of higher aeration shows that respiration is not directly limited by the immediate supply of available oxygen. Oxygen uptake by *P. chrysogenum* is unaffected by oxygen concentration until very low concentrations are reached. By plotting the rate of uptake of oxygen from solution by mycelium in a sample of liquor in a polarograph cell a concentration below 10 % of the value for saturation with air was reached before the concentration of oxygen began to limit oxygen uptake. If respiration in fermentations with low aeration were limited by the concentration of available oxygen, i.e. the latter being below the critical level, an increase in the concentration of oxygen would be expected to result in an increase in the respiration rate. Since this does not in fact occur, it must be assumed that the immediate concentration of available oxygen is not the limiting factor in respiration. In the fermentations with low aeration the concentration of available oxygen was adequate to permit a higher respiration rate than actually occurred. In such fermentations with low aeration, however, not only was the respiration rate diminished but also the quantity and rate of penicillin production. Thus for optimum penicillin production the rate of solution of oxygen in a vessel must not only ensure that the supply of oxygen is not limiting *respiration*, since very low concentrations of oxygen ensure this. Respiration is apparently limited by the magnitude of the existing respiratory mechanism. The latter appears to be synthesized in proportion to the concentration of available oxygen during early growth. A high degree of aeration results in a high concentration of dissolved oxygen and it is this concentration that apparently determines the future rate of respiration and possibly the rate of penicillin production also.

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The Extracellular Bacteriolytic Enzymes of a Species of *Streptomyces*

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SUMMARY: A bacteriolytic protease produced by a species of *Streptomyces* is activated by manganese and other metals. This enzyme digests autoclaved *Bacterium coli* but not autoclaved *Staphylococcus aureus*, though it does digest 'proteins' extracted from staphylococci with hot alkali. A ribonuclease is produced by the *Streptomyces* sp. but it apparently takes no part in the lysis of *Staph. aureus*. During lysis autoclaved *Bact. coli* do not disintegrate but progressively diminish in size.

Culture filtrates of actinomycetes are known to lyse heat-killed bacteria, Gram-negative organisms being far more readily lysed than Gram-positive ones (Waksman, 1947; Welsch, 1947; Jones, Swallow & Webb, 1948). Tai & van Heyningen (1951) showed that a species of *Streptomyces* produced at least two proteolytic enzymes, the action of which could account for all the lysis of heat-killed Gram-negative organisms. One of these enzymes acted upon casein and the other had a greater affinity for protein(s) present in *Bact. coli*, but the caseinolytic enzyme may also have been bacteriolytic. The possibility that other, non-proteolytic, enzymes were concerned in bacteriolysis could not be excluded, although polysaccharases were apparently not involved. Heat-killed Gram-positive organisms were far less susceptible to lysis by the streptomyces enzymes. Jones *et al.* (1948) suggested that such organisms are lysed by proteolytic enzymes only after they have been made Gram-negative by the action of ribonuclease. In support of this suggestion they demonstrated that the filtrates of 35-day cultures of an *Actinomyces* sp. contained a ribonuclease and that lysis of Gram-positive organisms was brought about only with these filtrates and not with those obtained from 50-day cultures which did not contain ribonuclease. They did not show that ribonuclease actually participated in the lysis, nor did they show that the actinomyces ribonuclease changed the organisms from Gram-positive to Gram-negative during lysis. Dubos (1937) showed that pneumococci which had been lysed by crystalline trypsin to the extent of losing 75 % of their weight and 92 % of their nitrogen were still Gram-positive entities, and Welsch (1947) found that heat-killed staphylococci could be lysed by the enzyme system of a *Streptomyces* sp. and still remain Gram-positive. He also found that staphylococci made Gram-negative by treatment with bile salts at 60° were no more sensitive to his enzyme system than untreated controls.

It has now been found that, of the two streptomyces proteases demonstrated by Tai & van Heyningen (1951), the one which readily attacks the protein(s) present in *Bact. coli* is activated by manganese and other metals, while the

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caseinolytic protease is inhibited by manganese and more or less unaffected by other metals. The lysis of a heat-killed Gram-positive organism (*Staph. aureus*) is not activated by manganese, although the digestion of proteins extracted from this organism with hot alkali is activated by manganese. Attempts to purify and separate these two proteolytic enzymes of this *Streptomyces* sp. have met with only limited success. A ribonuclease has been demonstrated in the culture filtrate but it does not appear to participate in the lysis of staphylococci. During lysis of Gram-negative organisms there does not appear to be any dissolution of the organism, but rather a diminution in size.

METHODS

Lytic preparations and estimations of lytic activities

The culture of *Streptomyces* used was that of Tai & van Heyningen (1951), and their methods were used for producing lytic filtrates, concentrating the lytic enzymes and determining bacteriolytic and proteolytic activities. Bacteriolytic activity was estimated by measuring the percentage lysis, i.e. percentage decrease in turbidity, of a standard suspension of autoclaved *Bact. coli*, caused by the lytic preparation in 15 min. under defined conditions. Casein and autoclaved *Bact. coli* were used as substrates for the proteolytic enzymes, and the activity of these enzymes was estimated by measuring the increase in trichloroacetic acid-soluble products, formed during digestion for 20 min. under defined conditions, by the spectrophotometric method of Kunitz (1947).

Ribonuclease estimations were made by a method described by Born (1951). It consists in digesting yeast ribonucleic acid with samples of the unknown ribonuclease and of a standard preparation of the enzyme and comparing the turbidities that are produced when the undigested nucleic acid is precipitated with HCl under defined conditions. The method is not described since little use was made of it in the work to be reported here.

Other determinations. Bacterial counts were done in a Helber chamber with dark-ground microscopy. Nitrogen was determined by the micro-Kjeldahl method. Values of pH were determined electrometrically with a glass electrode. Manganese was determined by the method of Stockdale & Dexter (1938).

RESULTS

Comparison of the rates of lysis of Gram-positive and Gram-negative organisms

Suspensions of autoclaved *Bact. coli* and *Staph. aureus* (0·63 mg. dry wt./ml.) were treated with the streptomyces enzyme preparation or with crystalline trypsin (30 μg./ml.) for 30 min. at 37° and the rates of lysis compared. (According to Vendrely (1946) the protein contents of coliform bacilli and staphylococci are both about 75 % of the dry weight.) Table 1 shows that the streptomyces preparation lysed the Gram-negative organism 3 times as fast as the Gram-positive and that with trypsin the difference in rates was 2-fold.

When a lower concentration of the streptomyces enzymes acted upon these substrates for a longer time the difference in rates of lysis of Gram-negative and Gram-positive organisms was not so great, as can be seen in Table 2. (These experiments were carried out with aseptic precautions.)

Table 1. *Lysis of autoclaved* Bact. coli *and* Staph. aureus *by a streptomyces preparation and by crystalline trypsin* (30 *min. at* 37°)

Substrate	Lysis (%) with Streptomyces preparation	Trypsin
Bact. coli	77	50
Staph. aureus	24	25

Table 2. *Lysis of autoclaved* Bact. coli, Staph. aureus *and* Strep. pyogenes *by a smaller concentration of the streptomyces preparation than that used in the experiments shown in Table* 1

	Lysis (%) of		
Time (hr.)	*Bact. coli*	*Staph. aureus*	*Strep. pyogenes*
4	62	43	23
16	80	70	61
32	85	74	66

Table 3. *Effect of various metal ions* (*at* 0·002 M) *on the proteolytic digestion of autoclaved* Bact. coli *by the streptomyces preparation*

Metal	Ratio: $\frac{\text{activity}+\text{metal}}{\text{activity}-\text{metal}}$
Manganese	3·44
Magnesium	2·10
Iron	1·45
Calcium	1·44
Copper	1·16
Cobalt	1·00
Zinc	0·98

Activation of bacteriolysis by metals

The effect of various metal ions on the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation was investigated. The effect of metals on the lysis of autoclaved *Bact. coli* could not be studied because addition of metal salts to the bacterial suspension caused variable changes in turbidity and interfered with the turbidimetric assay of bacteriolysis. Table 3 shows that manganese and some other metals had a considerable activating effect on the proteolysis of autoclaved *Bact. coli*.

Although emission spectroscopy (kindly carried out by Dr F. M. Brewer) showed no traces of manganese either in the streptomyces enzyme preparation or in the autoclaved *Bact. coli* substrate, various metal-binding compounds partially inhibited the action of the enzyme when no metals were added (Table 4). This inhibition may have been due to the removal of traces of other activating metals, such as magnesium or calcium, which were shown to be present.

Table 4. *Effect of metal-binding substances on the digestion of autoclaved* Bact. coli *by the streptomyces preparation*

Substance	Concentration (M)	Ratio: $\frac{\text{activity}+\text{substance}}{\text{activity}-\text{substance}}$
Ethylenediamine tetra-acetate	0·005	0·40
Citrate	0·10	0·52
Thiolacetate	0·0025	0·54
Cysteine	0·0025	0·70
Oxalate	0·000125	0·80

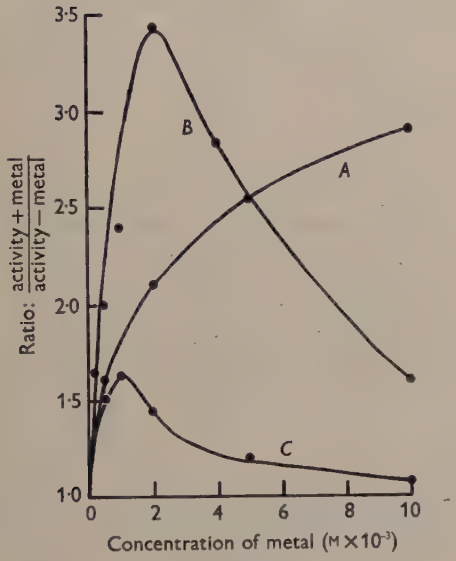

Fig. 1

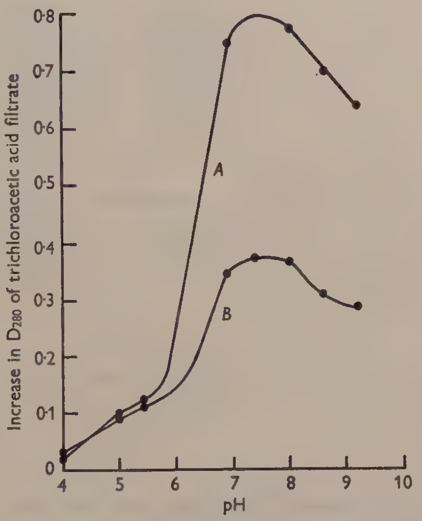

Fig. 2

Fig. 1. Activation of the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation in the presence of magnesium (*A*), manganese (*B*) and calcium (*C*).

Fig. 2. Effect of pH on the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation in the presence (*A*) and in the absence (*B*) of manganese.

Fig. 1 shows that the optimum concentration of manganese for the proteolysis of autoclaved *Bact. coli* was about 0·002 M. Fig. 2 shows that manganese did not affect the optimum pH for this proteolysis. Dialysis for 24 hr. decreased the concentration of manganese from 0·002 M to a level too low to estimate and completely reversed the activation. The effect of manganese on the digestion of several other proteins besides autoclaved *Bact. coli* was tested, with the results shown in Table 5. It is interesting to note that it was only the proteolysis of autoclaved *Bact. coli* which was activated, whereas the digestion of several other proteins, including casein, was inhibited. Other metals did not affect the digestion of casein.

Table 6 shows that manganese had no effect on the proteolysis of autoclaved *Bact. coli* by a crystalline protease of *Aspergillus oryzae* (kindly supplied by Dr W. G. Crewther; see Crewther & Lennox, 1950) or by crystalline trypsin.

At this stage it was clear that the manganese-activated enzyme in the streptomyces preparation was that which acted upon the protein(s) present in autoclaved *Bact. coli* and that several substrates from sources other than bacteria were not attacked by it. It was, therefore, interesting to find out whether this enzyme also acted on proteins of Gram-positive organisms.

Table 5. *Effect of manganese* (0·002 M) *on the digestion of various proteins by the streptomyces preparation*

Substrate	Ratio: $\frac{\text{activity}+\text{Mn}}{\text{activity}-\text{Mn}}$
Autoclaved *Bact. coli*	3·44
Denatured fibrinogen	0·89
Denatured haemoglobin	0·70
Denatured serum albumin	0·60
Denatured globin (from haemoglobin)	0·53
Casein	0·40

Table 6. *Effect of manganese* (0·002 M) *on the digestion of autoclaved* Bact. coli *by protease from* Aspergillus oryzae *and by trypsin*

Enzyme	Final concentration of enzyme (μg./ml.)	Ratio: $\frac{\text{activity}+\text{Mn}}{\text{activity}-\text{Mn}}$
A. oryzae protease	10	1·00
Trypsin	5	0·81

Suspensions of autoclaved *Bact. coli* and *Staph. aureus* (0·5 % dry wt./vol.) were treated with the streptomyces enzyme preparation with and without the addition of 0·002 M manganese, for 20 min. in the case of *Bact. coli* and for 60 min. in the case of *Staph. aureus*. After these times the mixtures were precipitated with an equal volume of 10 % (w/v) trichloroacetic acid and the ultraviolet absorption spectra of the filtrates determined. These showed that whereas the digestion of *Bact. coli* was activated by manganese there was no activation in the case of *Staph. aureus* (Fig. 3).

These results suggested that Gram-positive organisms are lysed more slowly than Gram-negative organisms because the manganese-activated enzyme does not participate in the proteolysis of the former. The results do not necessarily mean that Gram-positive organisms do not contain the substrate of the manganese-activated enzyme. It is possible that the substrate is present but protected from the action of the enzyme. Some light is shed on this point by the following experiment. Autoclaved *Staph. aureus* suspensions were treated with 0·5 N-NaOH for 20 min. at 56° and at 100° and centrifuged. The insoluble residues and the supernatants were neutralized and dialysed for 48 hr. against running tapwater and then digested with the streptomyces preparation with and without 0·002 M manganese. The results are shown in Table 7. Proteolysis of fractions obtained at 56° was unaffected by manganese, but the fractions obtained at 100° were readily digested by the manganese-activated enzyme.

Whether this was due to the destruction or removal of an inhibitor or to an alteration of the protein was not decided.

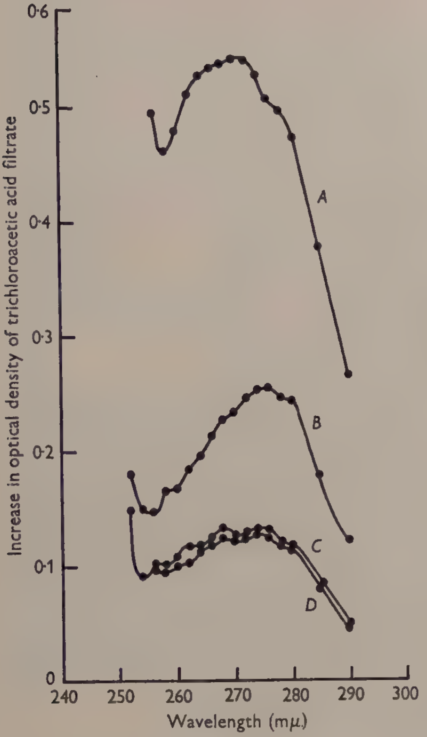

Fig. 3

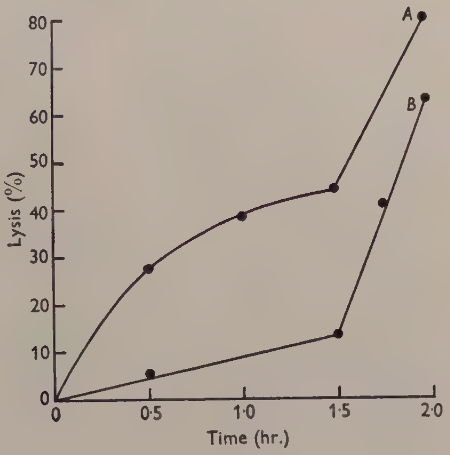

Fig. 4

Fig. 3. Ultraviolet absorption spectra of trichloroacetic acid-soluble material liberated by the streptomyces preparation from autoclaved *Bact. coli* in the presence (*A*) and in the absence (*B*) of manganese, and from autoclaved *Staph. aureus* in the presence (*C*) and in the absence (*D*) of manganese.

Fig. 4. Lysis of autoclaved *Staph. aureus* by ribonuclease (curve *A* from 0 to 1·5 hr.) and the streptomyces preparation (curve *A* from 1·5 to 2 hr.), and by the streptomyces preparation alone (curve *B* from 1·5 to 2 hr.).

Table 7. *Effect of manganese* (0·002 M) *on the proteolysis of fractions obtained from autoclaved* Staph. aureus *by treatment with* 0·5 N-NaOH *for* 20 *min. at* 56° *and* 100°

Temperature (°)	Fraction tested	Non-dialysable nitrogen in fraction (% of total)	Ratio: activity + Mn / activity − Mn
56	Insol. residue	67·5	1·1
	Extract	32·5	1·1
100	Insol. residue	12·0	3·4
	Extract	88·0	3·9

Attempts to purify and separate the streptomyces proteases

Numerous attempts were made to separate and purify the streptomyces enzymes by salting out and adsorption, but they met with little success. An attempt was also made to take advantage of the fact that the manganese-activated enzyme was more strongly adsorbed on to autoclaved *Bact. coli* than the caseinolytic enzyme (see Tai & van Heyningen, 1951). The mixture of enzymes was adsorbed with autoclaved *Bact. coli*, the organisms were suspended in buffer and incubated at 37° until they had all dissolved, and the resultant solution was again adsorbed with autoclaved *Bact. coli*. When this process was repeated once more a 15-fold concentration of the manganese-activated enzyme relative to the caseinolytic enzyme was obtained. However, this procedure had no practical value as the yields of enzyme were extremely low, and although there was a separation of the enzymes there was no increase in their activity per unit weight of non-dialysable nitrogen.

Many attempts were made to purify the enzymes by fractional precipitation with organic solvents under controlled conditions of ionic strength, temperature, pH, protein concentration and solvent concentration. The two enzymes were not separated and at best a 4-fold increase in total enzyme activity was obtained when the enzymes were precipitated with 80 % methanol (v/v final concentration) at pH 4·0, temperature −5°, and at an ionic strength equal to that of tapwater (about 0·005). With 80 % methanol the yield of both enzymes was about 80 %; with 40 % methanol the same purification was obtained, but the yield of enzymes was 25 %. The great solubility of both enzymes and their associated impurities in concentrated $(NH_4)_2SO_4$ solutions and in ethanol, methanol, and acetone prevented their purification.

The role of ribonuclease in the lysis of Gram-positive organisms

The preparation obtained by precipitating the streptomyces enzymes with 80 % methanol at pH 4·0 had ribonuclease activity (per mg. non-dialysable nitrogen) equivalent to 1/2000th of that of a sample of crystalline pancreatic ribonuclease (kindly supplied by Prof. J. N. Davidson), and the yield of ribonuclease in this precipitate was at least 50 % of that contained in the crude streptomyces culture filtrates. Jones *et al.* (1948) suggested that the lysis of Gram-positive organisms by actinomyces proteases was preceded by the removal of the Gram-staining material by ribonuclease. This could not be confirmed with the streptomyces preparation. When suspensions of autoclaved *Staph. aureus* (containing 2–3 % of Gram-negative cells) were lysed to the extent of 50 % by the streptomyces preparation there was no change in the Gram-staining properties of the residual cells, and the trichloroacetic acid-soluble products absorbed ultraviolet light with a maximum at about 277 mμ. which is characteristic of proteins (see Fig. 3). On the other hand, when these cells were treated with small concentrations of crystalline pancreatic ribonuclease the trichloroacetic acid fraction absorbed intensely at 258 mμ. Crystalline ribonuclease did cause some decrease in the turbidity of a suspension of autoclaved *Staph. aureus* (see Fig. 4), but the concentration of ribonuclease required to cause 25 % lysis was about 40,000 times as high as that in a streptomyces protease preparation that would cause the same amount of lysis in the same time.

Table 8. *Decrease in size of autoclaved* Bact. coli *cells during incubation with the streptomyces preparation*

(Temperature of incubation 37°.)

Time of incubation (min.)	Lysis (%)	Number of cells/ml. suspension	State of cells
0	0	$2{\cdot}5\times10^9$	Normal
20	38	$2{\cdot}6\times10^9$	—
45	58	$2{\cdot}6\times10^9$	—
60	63	$2{\cdot}5\times10^9$	Smaller, not elongated

Reduction in size of cells during bacteriolysis

When autoclaved *Bact. coli* cells were partially lysed by the streptomyces preparation there was apparently no dissolution of the cells but rather a diminution in size. Table 8 shows that after 63 % lysis there was no appreciable decrease in the total number of cells. Dubos (1937) obtained similar results when Gram-positive cells (heat-killed pneumococci) were treated with trypsin.

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Induced Variations in a Penicillin-resistant Staphylococcus

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SUMMARY: When young cells of a strain of a staphylococcus were exposed to substances noxious to the organism, variations transmissible to progeny were obtained. The variations consisted mainly in a decrease in antibiotic resistance and mouse virulence. Characters of pathogenic strains such as coagulase production, mannitol fermentation and gelatin liquefaction were also abolished.

The experiments were carried out in a way that excluded the selection of spontaneous mutants. Adaptation should also be excluded as all variations consisted in a loss or decrease of activities.

The variants when first isolated seemed unhealthy, the largest part of their progeny being non-viable and consisting of cells which grew up to a point and lysed without dividing. The results obtained were therefore attributed to a damage of the parent cell caused by the injurious agents used.

Antibiotics and other substances noxious to bacteria can induce changes in bacterial cells which are transmissible to progeny for a variable number of generations. The changes have been attributed to a profound disturbance or injury of the parent cell, and in one instance it had been possible to follow, step by step, the evolution of the disturbed cells (Voureka, 1951).

The present paper deals with a strain of staphylococcus in the progeny of which decrease of virulence and of resistance to penicillin and to streptomycin were noticed after young growing cells were exposed to injurious agents.

New biological characters appeared in a few or in all the colonies grown from the treated cultures. In some cases, the new characters remained unchanged through over 50 passages in normal laboratory media, while in others the variant cell acquired one or more of the characters of the parent.

EXPERIMENTAL

To exclude, as far as can be done, the possibility that the effects obtained were due to selection of spontaneous mutants endowed with the new characters, the experiments were planned as illustrated schematically in Fig. 1.

Freshly isolated cultures derived from one pair of cocci were used in order to have as uniform a population as possible, and the experiments were carried out on a small number of cells so that all the colonies from both the control and the treated bacterial suspension could be examined.

The strain selected was a coagulase-positive, phage typable, mouse virulent strain of *Staphylococcus aureus*, fermenting mannitol, liquefying gelatin, producing penicillinase and giving a clear-cut zone of haemolysis on horse blood agar; it was resistant to 400 units of penicillin and to 10 units of streptomycin; it had been kept in artificial media for a long time, had a sticky consistency and the originally aureus pigment was, at the time of its use, creamy.

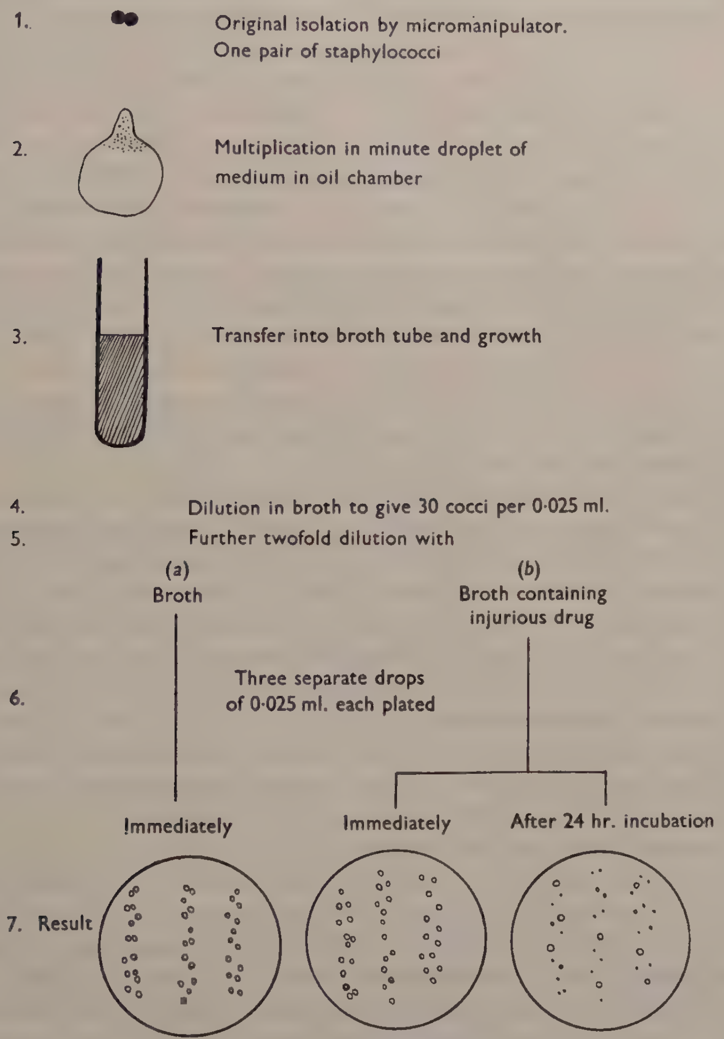
1. Original isolation by micromanipulator. One pair of staphylococci
2. Multiplication in minute droplet of medium in oil chamber
3. Transfer into broth tube and growth
4. Dilution in broth to give 30 cocci per 0·025 ml.
5. Further twofold dilution with
(a) Broth
(b) Broth containing injurious drug
6. Three separate drops of 0·025 ml. each plated
Immediately
Immediately
After 24 hr. incubation
7. Result

Fig. 1. Scheme of experiments.

Chloramphenicol as the injurious agent

A loopful of the freshly isolated culture was seeded into broth, left to grow for 3 hr. and then diluted in broth so that there were *c.* 30 cells/0·025 ml. To 0·1 ml. lots of this microbial suspension were added: (i) 0·1 ml. of broth containing 40μg. of chloramphenicol, and (ii) 0·1 ml. of broth. After vigorous shaking three 0·025 ml. lots of each mixture were each allowed to run over the surface of a blood agar plate thus reducing the concentration of the drug sufficiently to allow growth. Both mixtures and plates were incubated for

24 hr. The plate from mixture (i) served as a control of the mixture of chloramphenicol and bacterial suspension before the drug had time to act. The plate from tube (ii) was an additional control for the suspension of staphylococci in broth. Each 0·025 ml. lot yielded *c.* 15 well-separated colonies which all proved to have exactly the same characters as the parent culture.

After 24 hr. incubation the broth mixture containing chloramphenicol, in which no trace of growth was visible, was again vigorously shaken and three lots of 0·025 ml. were plated as before. The number of colonies recovered was usually less than that in the control plates. In most experiments, all, or at least some of the colonies had altered in character. Those which did not produce haemolysis on blood agar, or which showed gross divergence in colour or morphology from the control colonies were not further considered.

In some experiments the bacterial cells were exposed for a shorter time (6 hr.) to higher concentrations of the drug (100–125 μg./ml.) with similar results.

Repeated experiments with chloramphenicol gave fairly constant results when the bacterial cells were exposed to high concentrations of the drug for a period which was just sublethal.

The viable counts suggest that the concentrations used had inhibited completely the division of the cells. In order to exclude the possibility that there might have been continuous division which was balanced by an equivalent number of deaths, 1–10 pairs of staphylococci were isolated in the oil chamber of the de Fonbrune micromanipulator in droplets of drug-free nutrient medium as controls, and in the same medium containing 20 and 100 μg. chloramphenicol/ml. The medium was a filtrate of a broth culture of the original strain enriched with serum. Pairs of staphylococci were used in all manipulations instead of single cocci because the strain grew as diplococci, and any attempt to separate the pair resulted in some injury which often prevented multiplication. The preparations were held continuously at 37°, and observed by phase-contrast microscopy.

After 24 hr. incubation there was no division in most of the drug-containing droplets but the cocci grew larger; on rare occasions one more pair appeared in the first 2 hr. without any further division. When transferred to drug-free medium some of the treated cells grew. Biological changes, similar to those described in Tables 1 and 2, were observed in two out of the three colonies yielded by a droplet containing five diplococci that had been exposed for 24 hr. to 100 μg. of chloramphenicol. The controls grew profusely.

Other injurious agents

Many experiments were done in exactly the same way, using penicillin in place of chloramphenicol. A few were also done using terramycin, nitrogen mustard and hydrogen peroxide. With all these substances the variants isolated were similar to those induced by chloramphenicol.

As shown in Table 1, when cells resistant to 400 units penicillin/ml. were exposed to high concentrations of the drug (50,000 units/ml.) for a sublethal time, cells were isolated which were highly sensitive to penicillin. These experiments were repeated many times with the same results. Since the concentration of penicillin was very high, penicillinase was added to the mixtures before plating and there was an additional control consisting of the suspension of the cells and penicillinase alone.

RESULTS

In each experiment all the colonies on the control plates were examined and were all found to have the characters of the parent strain.

Most of the variants from the treated suspensions had greatly decreased resistance to penicillin and to streptomycin, whereas there was no change in their sensitivity to chloramphenicol. Their growth was very poor, but it was markedly improved in well-aerated thin layers of broth, while the growth of the parent strain was also improved but to a much less extent. The cells had apparently acquired a greater need for oxygen and autolysed more readily in the usual broth cultures. The variant cells no longer produced coagulase; they were not attacked by the phages which lysed the parent strain nor by any of the phages normally used for typing coagulase positive strains. Their capacity to ferment mannitol, to liquefy gelatin and to produce penicillinase was also lost, but they retained their creamy pigment and were haemolytic on blood agar. One variant (variant IV) had suffered less; it remained very resistant to penicillin, although less so than the original strain, retained some resistance to streptomycin, grew well, produced coagulase and fermented mannitol, though rather slowly, and was attacked by the same phages as the original strain, though to a less extent. It should be noted that in all tests the inoculum of the variants was much greater than that of the parent strain to allow for the poorer growth of the former. All results were read after 48 hr. incubation.

Tables 1 and 2 give details of some characters studied in representative variant types on their 5th subculture on drug-free media as compared with those of the control.

Variants II–IV were subcultured 50 times in drug-free media. As a result, their growth improved; the resistance to penicillin of variants II and III increased twofold while all other characters remained unchanged; and variant IV became indistinguishable from the original strain except that it remained less resistant to penicillin and streptomycin.

All variants (except VI and VII which died) were retested after repeated subcultures over 3 months. During this period the resistance to penicillin of some variants had partly reverted. Although this resistance was in some cases considerable (100 units of penicillin/ml.) no detectable penicillinase was produced. There was also a slight reversion of the resistance to streptomycin. Coagulase activity did not reappear and phage typing was not attempted (see Table 3).

The virulence of the original strain and of the variants was compared in mice. Mice were injected intravenously (Gorrill, 1951) with 1 ml. of a thick suspension (24 hr. agar slope culture suspended in 2 ml. of saline). Four mice

Table 1. *Drug-produced variants in a strain of* Staph. aureus *and their biological divergence from the parent strain on their fifth subculture on drug-free media*

						Degree of resistance. Least concentration where growth occurs		
Variants	Drug used	Concentration of drug used	Time of exposure (hr.)	Growth in broth	Phage type	Penicillin (u./ml.)	Strepto-mycin (u./ml.)	Chlor-amphenicol (μg./ml.)
I	Chloramphenicol	125 μg./ml.	6	+	–	0·03	0·6	nt
II	Chloramphenicol	20 μg./ml.	24	+	–	0·03	1·5	3
III	Chloramphenicol	20 μg./ml.	24	+	–	0·25	2·5	3
IV	Chloramphenicol	20 μg./ml.	24	+ + +	47 +	100	0·6	nt
V	Chloramphenicol	125 μg./ml.	6	+	–	0·03	2·5	nt
VI	Penicillin	50,000 u./ml.	6	+	–	0·03	1·25	nt
VII	Penicillin	50,000 u./ml.	6	+ +	–	0·03	1·25	nt
VIII	Terramycin	50 u./ml.	6	+	–	0·03	0·6	nt
IX	Terramycin	12·5 u./ml.	6	+	–	0·12	1·5	3
X	Nitrogen mustard	2·5 mg./ml.	24	+ +	–	0·12	1·5	3
XI	Nitrogen mustard	2·5 mg./ml.	24	+ +	–	1·0	1·5	3
XII	Hydrogen peroxide	40 mg./ml.	6	+ +	–			
XIII	Hydrogen peroxide	3 mg./ml.	24	+	–	0·50	1·5	1·5
Parent strain	0	.	.	+ + +	47/47*c*/53 +	400	10	3

All variants were coagulase negative except variant IV which showed delayed coagulation. They all showed same pigment production and haemolysis on horse blood agar as the parent strain.

nt = not tested; – = no reaction obtained.

Table 2. *Sugar fermentations of variants and parent strain*

Variants	Lactose	Glucose	Dulcitol	Mannitol	Sucrose
I	+	+	–	–	+
II	+	+	–	–	+
III	+	+	–	–	+
IV	+	+	–	±	+
VI	+	+	–	–	+
VII	+	+	–	–	+
VIII	+	+	–	±	+
IX	+	+	–	–	+
X	+	+	–	–	+
XII	+	+	–	–	+
XIII	+	+	–	–	+
Parent strain	+	+	–	+	+

+ = acid formation; ± = delayed acid formation and weaker reaction.

were used for each variant, and in none was any ill effect noticed. Heart blood culture 3 days after inoculation proved to be sterile. Ten mice were inoculated each with 0·5 ml. of the parent strain in a suspension turbidimetrically one-tenth as dense. Of these eight died in 24 hr. and two became very ill and were killed on the third day; the infecting organism was recovered from the heart-blood of all ten animals.

Table 3. *Comparison of biological characters of the variants and parent strain after repeated subculture on drug-free media over 3 months*

				Degree of resistance. Least concentration where growth occurs	
Variants	Growth	Coagulase production	Phage type	Penicillin (u./ml.)	Streptomycin (u./ml.)
I	++	−	nt	0·06	0·3
II	++	−	nt	3	0·3
III	++	−	nt	25	0·6
IV	+++	+	47/47*c*/53/54+	400	2·5
VII	++	−	nt	0·03	0·6
VIII	++	−	nt	0·25	0·6
IX	++	−	nt	0·03	0·3
X	++	−	nt	3	2·5
XI	++	−	nt	6	0·3
XII	++	−	nt	50	1·25
XIII	++	−	nt	100	0·3
Parent strain	+++	+	47/47*c*/53+	400	2·5

All variants showed same pigment production and haemolysis on horse blood agar as the parent strain. All except variant IV and the parent strain were coagulase negative.

nt = not tested.

DISCUSSION

All the colonies on the control plates were found to have the characters of the parent strain. This seems to exclude as far as possible that the variants were present in the original culture. Sometimes all, and usually a large proportion, of the colonies obtained from the fifteen treated cells were altered. The ratio of altered to unaltered cells is thus in all cases greater than 1:15 and can hardly be that of spontaneous mutants appearing in the treated suspension and isolated through some kind of selection.

The poor and slow growth of most of the variants when first isolated, which made necessary the use of very large inocula and of longer incubation to assure growth in subsequent subcultures, also makes it improbable that these rather unhealthy cells would have survived selectively under adverse conditions and that these conditions should be presumed to favour their selection.

On repeated subcultures the growth of the variants improved. No study of these poorly growing cells and of their progeny in single cell cultures was made on this occasion. Most probably, however, the process is the same as that with variant β of *Bacterium coli* D/433 (Voureka, 1951); that is, a large part of the progeny of the treated cell is non-viable, consisting of members which grow and lyse without dividing. When a small inoculum is used the culture may not survive, but with subcultures of large inocula on drug-free media the healthiest individuals survive by selection, the viable proportion of the progeny gradually increases and the growth improves. Although the behaviour of the variant cell was not observed under the microscope, swollen cocci and lysed swollen cells formed the largest part of the population in the cultures of the variants when first isolated, and this feature often persisted through many subcultures. Here again, as with variant β of *Bact. coli* D/433 the bacteria with altered characters isolated after treatment seemed unhealthy.

All the drugs used produced similar variations in the coccus. It would seem that it is not a question of a specific mode of action of the drugs but of attaining a certain degree of disturbance of the bacterial cell, which, when reached, has the same results. It may be that virulence and resistance to drugs in concentrations higher than those the normal members of the genus would survive are labile characters and therefore more easily changed.

The results obtained with penicillin are of interest. They seem to contradict the usual conception that exposure to a sublethal dose of penicillin tends to increase the resistance of the organism to the drug. Many have observed that increased resistance can be induced by this method. Here, however, the conditions of the experiment are not the same. The ordinary method is to start with a sensitive staphylococcus and gradually to increase the concentration as the experiment proceeds. Here we start with a penicillin-resistant staphylococcus and after one exposure to 50,000 units/ml. for a sublethal time, resistance is reduced.

The variations seem to consist in a loss or a reduction of activities, i.e. virulence and characters associated with it, such as coagulase production, mannitol fermentation, gelatin liquefaction; there is also a loss of penicillinase production. The loss of so many activities, the poor and slow growth, the swollen lysing cells, suggest that the cells have been severely disturbed or damaged by the drugs used. The variants have been *caused* to appear; they were not present in the control suspension and it is improbable that they would have appeared on the control plates if the cells had been allowed to divide only once or twice, as might have been the case in the treated suspensions.

Some of the new characters were stable; it is possible that an injury to the parent cell, caused by the noxious agents used may have resulted in some permanent alteration in its genetical structure.

Assuming that the number of genes or their pattern rule the characters of a bacterial cell, an alteration in this number or pattern would result in alterations of characters. It is not inconceivable that such alterations may appear as a result of the inflicted injury. In the cases described the alteration was lethal for many members of the progeny of the disturbed cell; they seemed to lose the capacity to divide and died after reaching a certain stage of growth. Some other members, however, survived, kept the new genetical structure and the characters it involves as a permanent feature and transmitted them to their progeny.

One thus induced mutant would have the same genetic pattern as a so-called spontaneous mutant with the same characters; and, as in spontaneous mutations, in mutations deliberately induced by adverse conditions there should be a certain distribution of frequency of the mutants with a greater incidence for more labile characters, but in the latter the rate would be expected to be much greater.

It is possible that the only difference between spontaneous mutants and the alterations described in this paper is that while there is evidence to attribute the appearance of the latter to a lesion provoked by adverse conditions, it is not known why the former occur. It may be that spontaneous mutants also appear because of an occasional disturbance to a cell which causes it to produce among its progeny a few members with altered characters.

In a previous paper (Voureka, 1948) I showed that resistant staphylococci could be rendered sensitive to penicillin by growth with a strain of a haemolytic streptococcus. It is possible that a similar mechanism was then involved, the active streptococcus releasing substances poisonous to the staphylococci. Unfortunately, the streptococcus died before it could be thoroughly studied. Nevertheless, after mixed growth the staphylococcus recovered was either wholly sensitive or consisted of a population in which the sensitive members were far more numerous than in the controls. (The strains of staphylococcus used were genetically unstable, spontaneously producing a number of sensitive variants; Barber, 1948.)

The growth in mixed cultures had in some way either induced the appearance in the progeny of the treated bacteria of a larger number of penicillin-sensitive members or had selectively killed the penicillin-resistant ones. There can hardly be a third alternative.

CONCLUSIONS

Permanent variations can be induced in staphylococci. The conditions inducing these variations and the fact that they consist mainly in loss or decrease of activities suggest that the process through which they appear is neither a selection nor an adaptation but a result of cell damage.

When virulence was lost, other characters associated with virulence were also lost. If the variations are due, as is suggested, to an induced change of the genetic pattern or of the nuclear constitution, the simultaneous loss of all these characters might mean that they have the same or closely related genetic location.

The fact that these 'unhealthy' cells deprived of so many characters have also lost resistance to certain drugs suggests that resistance to these drugs, at least when not normal to the species, is more exacting than sensitivity, that is, involves some additional capacity.

I wish to thank Sir Alexander Fleming for his advice, help and encouragement; Prof. R. Cruickshank for his interest and suggestions; Dr R. E. O. Williams and Miss Joan Rippon (Central Public Health Laboratory, Colindale) for all phage typings; my colleague, Dr R. H. Gorrill, for helpful discussion, and Mr Ben May, Alabama, for financing this work.

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The Auxanographic Responses of certain Aneurin-requiring Yeasts to Aneurin, Aneurin Components and Derivatives

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SUMMARY: Three yeast species, *Rhodotorula mucilaginosa*, *Trichosporon cutaneum* and *Torulopsis albida* (*Cryptococcus albidus*) respond in a simple auxanographic spot test to the presence of aneurin, aneurin split products and cocarboxylase. Differences in the behaviour of these three species towards the different biologically active entities make it possible to differentiate between these substances and to identify them. Moreover, from the appearance of the auxanogram conclusions can be drawn regarding certain dynamic aspects of aneurin metabolism such as velocity of uptake, storage and splitting of aneurin, or of its derivatives. The test is not only qualitative but also, within certain limits, quantitative.

An auxanographic procedure for the determination of vitamin requirements of yeasts by means of drops of vitamin solutions applied with a bacteriological loop has been described earlier (Mager & Aschner, 1947). It was noted at that time that the diameter of the growth field was roughly proportional to the concentration of the vitamin in the drop applied. As the preparation of the auxanogram is very simple and the amount of test solution needed extremely small (a loopful being quite sufficient) it seemed desirable to examine how far this procedure could serve as a method for quantitative or semi-quantitative determination of a vitamin. We were especially interested in the determination of aneurin because, with suitable yeasts as test organisms, a much simpler test medium could be employed than the one proposed in the auxanographic assay method of Bacharach & Cuthbertson (1948) where a lactobacillus serves as the test organism.

Our conclusions are based on the comparative study of the auxanographic responses of three aneurin-requiring organisms towards aneurin, aneurin pyrophosphate (cocarboxylase) and two synthetic compounds representing respectively the pyrimidine and the thiazole moieties of the aneurin molecule. Different biologically active split products of aneurin served as controls.

MATERIALS AND TECHNIQUE

Experiments were carried out with three yeast species belonging to three different genera (nomenclature according to Lodder, 1934, and Diddens & Lodder, 1942): *Rhodotorula mucilaginosa*, *Trichosporon cutaneum* and *Torulopsis albida* (*Cryptococcus albidus*). Some experiments were also undertaken with *T. neoformans* (*Cryptococcus neoformans*) which behaved in every respect like *T. albida*. The strains were kept as stock cultures on a glucose peptone Marmite medium and were transferred 24 hr. before the test on to a medium of the following composition: glucose, 1·0 g.; $(NH_4)_2SO_4$, 0·2 g.; K_2HPO_4, 0·1 g.; $MgSO_4$, 0·04 g.; agar (Difco), 2·0 g.; tapwater to 100 ml. The medium used for the auxanographic plates was of the same composition.

The materials tested were: aneurin chloride hydrochloride (U.S.P. reference standard); the synthetic pyrimidine moiety (2-methyl-5-ethoxymethyl-6-aminopyrimidine, Merck); the synthetic thiazole moiety (4-methyl-5-(β-hydroxyethyl)-thiazole, Merck) and aneurin chloride pyrophosphate (cocarboxylase, Merck). The following preparations of split products were compared with the synthetic compounds: aneurin treated at 120° at acid and alkaline pH; aneurin treated with fish 'thiaminase'; and aneurin which was in contact with resting yeast cells. All these substances, and combinations of them, were usually tested in the range of 0·01–2 μg./ml.

The auxanographic plates were prepared in the following way. To a test-tube containing 6 ml. of melted agar cooled to 50°, 0·5 ml. of a suspension of test organism was added as an inoculum and the plates poured after quick but thorough mixing. Care was taken to have an even thickness of the agar layer all over the plate. Therefore only plates with perfectly flat bottoms were used and these were kept on a water-level plane during solidification of the agar. For the inoculum a 24 hr. slope culture of test organism was washed into 3 ml. sterile distilled water. One half ml. of these washings contained about 50 million cells, an amount which in previous experiments was found to give satisfactory results. Before the application of the test solutions the plates were kept for 3 hr. at 30° in the incubator.

The test solutions were deposited as drops on the agar surface by means of a bacteriological platinum loop (diameter *c.* 3 mm.) which was flamed before each application. Six to ten solutions could be tested simultaneously on the same plate. The incubation temperature was 30°. The circular areas of growth responses became visible macroscopically after several hours' incubation and then remained practically unchanged in diameter. The microcolonies composing this growth, however, continued to grow for several days, so that the density within the growth area increased with time. Results were usually read after 20 hr. or more.

Quantitative estimations of aneurin were carried out by comparing on the same plate several dilutions of the material to be tested with a series of known solutions of aneurin in the range of 0·1–2 μg./ml.

EXPERIMENTAL

Auxanograms of aneurin, its pyrimidine and thiazole moieties and cocarboxylase

Auxanogram plates were prepared in the manner described above with the three test organisms, and drops of aneurin solutions of different strengths applied to the agar surface. Well-defined growth areas were obtained with concentrations in the range 0·05–2 μg./ml. After 20 hr. the growth within the positive areas was already so dense that, although composed of numerous microcolonies, it gave the impression of one single large colony. The density of growth appeared equal throughout the whole growth area, except for a small border zone, barely visible to the naked eye, where a gradual decrease in the size of the colonies could be observed (Pl. 1, fig. 1(1) and fig. 2(1)). Responses were also obtained with much lower concentrations such as 0·005 μg./ml. These areas of growth, however, were too poorly defined to serve for quantitative determinations and can only be referred to as traces. With higher concentrations some interfering phenomena were observed; for instance, an outer zone of sparser growth in the case of *T. albida*, and a transparent central area in the growth response of *R. mucilaginosa*. An explanation for these phenomena will be attempted later. With all three strains the size of the growth areas increased with increase in aneurin concentration applied. However, at least a twofold to fivefold ratio between different concentrations was necessary in order to produce measurable differences in the diameters of the growth areas. Table 1 contains actual measurements of the diameters of the auxanographic responses produced by aneurin solutions in varying concentration. No discernible differences in the sensitivity of the three different strains were noted.

Table 1. *Extent of the areas of auxanographic response of* R. mucilaginosa *and* Tr. cutaneum *resulting from the application of varying aneurin concentrations*

Aneurin (μg./ml.)	Diameter of growth area (mm.)
5	8
1	6
0·2	5
0·04	4

Mixtures of the pyrimidine and thiazole moieties, equimolar in concentration to the aneurin solution tested previously, gave areas of response which were always considerably larger than the corresponding aneurin auxanograms (Pl. 1, fig. 1(4) and fig. 2(4)). The ratio between the diameters of growth areas was about 2:1 in the case of *R. mucilaginosa* and 4:1 in the case of *Tr. cutaneum*. In addition to the larger diameters, the responses to mixtures of the two moieties showed another characteristic difference; the microcolonies composing the growth response remained smaller, and the transition zone from the positive to the negative area was much broader than in the corresponding aneurin auxanogram. The whole growth area was therefore less opaque and its outlines less sharply defined than in the case of aneurin (Pl. 1 figs. 3 and 4).

All three yeast species required for a threshold response a higher concentration of the moieties than of aneurin. The corresponding values in the case of *R. mucilaginosa* and *T. albida* were a twofold concentration, and in the case of *Tr. cutaneum* a fivefold concentration of the moieties (Table 2).

Further differences in the behaviour of the three yeast species became evident when they were tested against each of the two moieties separately: *R. mucilaginosa* reacted to the pyrimidine as to a mixture of both moieties, while *Tr. cutaneum* and *T. albida* gave no response with the pyrimidine alone even when the concentration was raised to 50 μg./ml. (Pl. 1, fig. 1(2) and fig. 2(2)).

When the ratio of the pyrimidine and thiazole moieties in the mixture was changed, the dimensions of the growth areas of *R. mucilaginosa* were affected only by the concentration of pyrimidine. Responses of *Tr. cutaneum* and *T. albida* were not perceptibly influenced by a preponderance of either of the two moieties.

The thiazole moiety in the absence of the pyrimidine moiety produced very weak responses with all three yeast species. With *R. mucilaginosa* responses remained small and faint even when 1 μg. thiazole/ml. was applied.

Table 2. *The lowest concentrations of growth-promoting substance (μg./ml.) necessary to produce a discernible response*

	μg./ml.				
Test organism	Aneurin	Pyrimidine moiety	thiazole moiety	Mixture of both moieties in the ratio of their molecular weights, pyrim. + thiaz.	Cocarboxylase
Rhodotorula mucilaginosa	0·005	0·005	0·04	0·005 + 0·004	2·7
Trichosporon cutaneum	0·005	—*	0·2†	0·025 + 0·021	0·14
Torulopsis albida	0·005	—*	0·2†	0·005 + 0·004	0·014

* No responses even at 50 μg./ml.

† These responses remain faint even following the application of 1 μg./ml.

With concentrations above 0·1 μg./ml. *Tr. cutaneum* and *T. albida* yielded rather extensive areas of growth which were, however, so weak as to be barely visible. The microcolonies in the centre were somewhat larger and so gave the impression of a more dense growth, but even the central part was considerably less opaque than the growth in the auxanogram of the mixture of both moieties (Pl. 1, fig. 1(3) and fig. 2(3)). With concentrations below 0·1 μg./ml. only the central part was visible.

By using an agar medium with 0·5 μg./ml. pyrimidine moiety for the auxanogram plate and *Tr. cutaneum* or *T. albida* as test organism it was also possible to produce well-defined growth areas on adding the thiazole moiety. Plates prepared in this way gave responses to a mixture of the two moieties which were dependent only on the thiazole concentration in the test solution. *R. mucilaginosa* of course always grew homogeneously throughout the whole plate under these conditions.

Mixtures of aneurin with its pyrimidine and thiazole moieties (0·5 aneurin + 0·25 pyrimidine + 0·21 thiazole, μg./ml.) produced auxanograms in which it was obvious that aneurin and the moieties produced their responses separately. The growth area was composed of a dense central part comparable with the response produced by aneurin alone and a sharply separated outer zone which was less dense, like the auxanogram of the moieties (Pl. 1, fig. 1(5) and fig. 2(5)).

Cocarboxylase in this range of concentrations gave no response with *R. mucilaginosa*, a clearly perceptible response with *T. albida*, and a definite but less sharply pronounced response with *Tr. cutaneum* (Pl. 1, fig. 1(6) and fig. 2(6)). Even concentrations as high as 50μg./ml. produced only traces of a response with *R. mucilaginosa*. Table 2 summarizes the lowest concentrations of all substances tested giving a discernible reaction.

Auxanograms of different split products of aneurin

Aneurin solutions which were autoclaved at 120° for 30 min. at pH 8·0 or 10·0 stimulated *R. mucilaginosa* as did solutions which contained the free pyrimidine moiety, i.e. they gave more extensive but less dense responses than the untreated control solution of aneurin. However, as the response areas with *Tr. cutaneum* and *T. albida* were not only weaker but also less extensive than with the aneurin control solution, we must conclude that the thiazole moiety was lacking entirely or was present only in traces. Indeed these two species gave no response at all to 1/5 dilutions of the treated solutions, whereas *R. mucilaginosa* responded well in this case. Beadle, Greenwood & Kraybill (1943) concluded from spectrophotometric data that the pyrimidine moiety was present in the split products of similarly treated aneurin. Our results concerning the pyrimidine moiety agree with this conclusion. Solutions of cocarboxylase treated in the same way likewise indicated the presence of the pyrimidine moiety alone. Heating a solution of aneurin in the autoclave at 120° at pH 5·0 also effected a cleavage of the aneurin molecule, as shown by the more extensive growth response of *R. mucilaginosa* in comparison with the response to the untreated aneurin solution. In this case, however, both *Tr. cutaneum* and *T. albida* yielded an increased area of growth, an effect which is brought about only when the thiazole moiety is present in addition to the pyrimidine moiety. We must assume therefore that, in contrast to the alkaline cleavage, at the acid pH the aneurin was split without simultaneous destruction of the thiazole moiety.

Aneurin, after 15 hr. contact with thiaminase prepared from carp intestine according to Krampitz & Woolley (1944), gave with *Tr. cutaneum* an extensive response indicative of the presence of a mixture of the two moieties, yet no response at all was given by *R. mucilaginosa*. According to the behaviour of *Tr. cutaneum* the two moieties of aneurin are present in the treated solution. As, however, *R. mucilaginosa* failed to indicate the presence of the pyrimidine moiety this substance was apparently present in a modified form which *Tr. cutaneum*, but not *R. mucilaginosa*, was able to utilize. Krampitz & Woolley (1944) had a similar experience in their assay with *Endomyces vernalis*. This organism, which is an indicator for the pyrimidine moiety gave only a very weak reaction in this case. They concluded that cleavage of aneurin by their thiaminase preparation produced the thiazole moiety and a derivative of the pyrimidine moiety.

Action of yeast cells on aneurin solutions

Uptake of aneurin by resting yeast cells was demonstrated in the following experiment. About 50 million cells of *Saccharomyces carlsbergensis* were suspended in 10 ml. of an aqueous solution (2μg./ml.). At intervals samples were taken and the suspended yeast cells were removed by centrifugation. The supernatant fluids were then analysed by the auxanographic method. The response areas became progressively smaller until after 1 hr. auxanograms of the supernatant fluids were negative with all three yeasts species. The missing aneurin was detected, by the thiochrome method, in the sedimented cells. The yeast cells obviously had taken up aneurin from the supernatant fluid. When a mixture of moieties was tested in the same way no perceptible decrease of concentration could be demonstrated in the supernatant. The uptake of split products by yeast cells is obviously much smaller than the uptake of intact aneurin.

An additional action of yeast cells on aneurin was observed by testing auxanographically aneurin solutions which had been in contact with yeast cells for several hours. Except for the longer contact period solutions were treated as in the two previous experiments. The result was a characteristic change in the auxanographic reaction of the supernatant. Auxanograms of successive samples revealed a perfect transition from the compact aneurin response to the diffuse response typical of the aneurin moieties (Pl. 1, fig. 5). A gradual splitting of aneurin had obviously taken place during the prolonged period of contact. Analysis, by the thiochrome method, of the sedimented cells and the supernatant fluid supported this assumption, in so far as a loss of aneurin in the system corresponding to the apparent increase in split products could be demonstrated. The splitting was produced with cells of *Saccharomyces carlsbergensis* or of *S. cerevisiae* but was much more pronounced when certain oxidative yeasts, especially *T. albida*, acted upon the aneurin solution. In this case first signs of splitting were evident after a contact period of 1 hr. A detailed description of these experiments will be given elsewhere.

DISCUSSION

The vitamin auxanogram has its counterpart in auxanography in the plate method for determination of antibiotic substances. Both methods are based on the same principle: interaction between a susceptible micro-organism, suspended in agar, and an active substance diffusing through this agar. The interaction itself, however, is different in the two cases, and this obviously also affects the diffusion process. In the inhibition response each cell that comes in contact with an effective concentration of the antibiotic is killed or inhibited without much further interaction with the diffusing substance. In the vitamin auxanogram, however, the relatively short contact between cell and growth factor is followed by a period of proliferation, which may continue for several days. This is possible only if a substantial part of the growth-promoting substance is stored by the test organism during the contact period. The capacity of yeast cells to store a large amount of different vitamins has indeed been demonstrated for various yeasts. This means that while in the inhibition auxanogram diffusion proceeds practically undisturbed, in the vitamin auxanogram it is highly modified by processes of absorption, storage, and use in the cells of the test organism. This might explain why a relatively great shift in concentration of vitamin (twofold to fivefold) is necessary to produce a perceptible change in the diameter of the growth area. The following observation supports this theory. There are differences in the extent of the growth areas when the same concentration of aneurin is applied on test plates containing different numbers of suspended yeast cells; the larger this number the smaller is the growth area.

The modifying influence of absorption and storage is probably also the reason for the differences in size of equivalent auxanograms of aneurin and aneurin split products. If the cells of the test organism are not able to store the moieties as such but have to transform them into aneurin before storage, they may collect a smaller amount of aneurin during a given period of contact than when aneurin itself is available in the surrounding medium. More substance of the two moieties would remain for diffusion, and the resulting growth would consequently be composed of smaller colonies over a larger area. Evidence for a smaller rate of uptake of split products by yeast cells was provided in our experiments with resting cells.

The transparent central areas in the auxanogram of *R. mucilaginosa* which appear usually when the aneurin concentration in the applied drop is higher than 2 μg./ml. are obviously due to inhibition of growth at this concentration. *R. mucilaginosa* seems to be more sensitive in this respect than, for example, *Tr. cutaneum* which gave homogeneous auxanograms even with 10 μg./ml. aneurin.

A different explanation is suggested for the reported interference of higher concentrations of aneurin in the case of auxanograms of *T. albida*. Here it is the outer zone of the auxanogram which is less dense than the central part. This condition is characteristic of response to a mixture of aneurin and split products. Evidently with higher concentration of aneurin a considerable part of the surplus aneurin is split, and it is the presence of split products which is responsible for the lack of homogeneity in the auxanogram. That the cleavage should be recognizable with *T. albida* but not with the other two species is not surprising, since *T. albida* is indeed more active in splitting aneurin than are other yeast species.

R. mucilaginosa, in contrast to *Tr. cutaneum* and *T. albida*, is unable to respond in the auxanogram to either diphosphorylated aneurin (cocarboxylase) or the pyrimidine derivative which results from cleavage of aneurin by thiaminase.

It seems that *R. mucilaginosa* lacks enzymes present in the other two species which liberate aneurin or its pyrimidine from combination. Cocarboxylase after treatment with takadiastase is a suitable growth factor for *R. mucilaginosa*, which then gives a typical aneurin response to the product of such treatment.

The use of aneurin auxanogram might be advantageous in several fields. For instance, with this method it is simple to follow the changes which occur in the aneurin content of a substrate during different stages of industrial processing, or to detect the appearance of aneurin split products in metabolic processes. These split products can be characterized to a certain degree and, in the absence of aneurin, may be estimated quantitatively. The pyrimidine moiety is determined by regular test plates with *R. mucilaginosa*; the thiazole moiety by using *T. albida* in test plates to which a suitable amount of pyrimidine moiety has been added. The quantitative estimation of aneurin itself is possible if no growth-promoting derivatives of aneurin are present, otherwise the values obtained might be too high. While it is obvious that the degree of accuracy and sensitivity of this simple test cannot reach that attainable by more elaborate methods, it is nevertheless surprisingly high. Moreover, the test permits the observation of subtle interactions between cell and vitamin which might be difficult to detect by other methods.

We are indebted to Merck and Co. Inc., (Research and Development Division), Rahway, New Jersey, U.S.A., for supplying us with the synthetic aneurin moieties and the cocarboxylase.

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EXPLANATION OF PLATE

Fig. 1. Auxanographic responses of *Rhodotorula mucilaginosa* to equivalent drops of solutions containing (μg./ml.): (1) 0·5 aneurin; (2) 0·25 pyrimidine moiety; (3) 0·21 thiazole moiety; (4) a mixture of both moieties in the ratio of their molecular weights and equivalent to 0·5 aneurin; (5) the same mixture as in (4) with the addition of 0·5 aneurin; (6) 0·7 cocarboxylase. (Magnification, × 0·7.)

Fig. 2. The same as Fig. 1 with *Trichosporon cutaneum* as test organism. Note the absence of response to the pyrimidine moiety in (2) and the response to cocarboxylase in (6). (Magnification, × 0·7.)

Fig. 3. Border area of the growth response of *R. mucilaginosa* to aneurin. The auxanogram has a dense structure and a well defined boundary. (Magnification, × 30.)

Fig. 4. Border area of the growth response of *R. mucilaginosa* to a mixture of thiazole and pyrimidine. Loose structure, boundary less distinct. (Magnification, × 30.)

Fig. 5. Auxanogram of four successively drawn samples of an aneurin solution (1μg./ml.) which was maintained in contact with cells of *Torulopsis albida*. Test organism: *R. mucilaginosa*. Samples were taken after the following periods of contact: (1), 0 (control); (2), 1 hr.; (3), 4 hr.; (4), 22 hr. The auxanograms reveal a gradual splitting of aneurin. (Normal size.)

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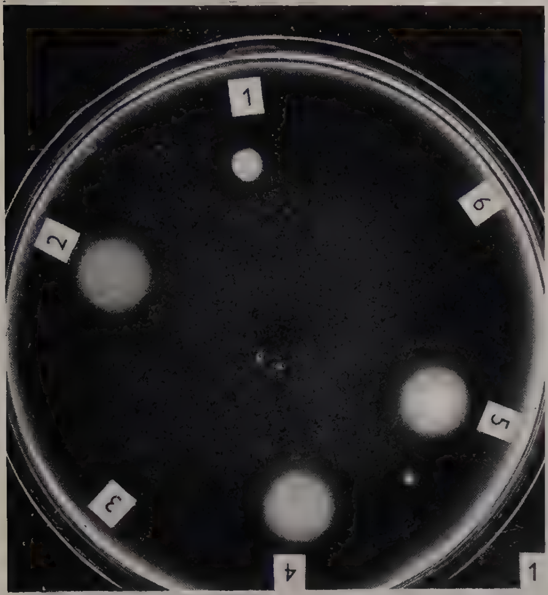

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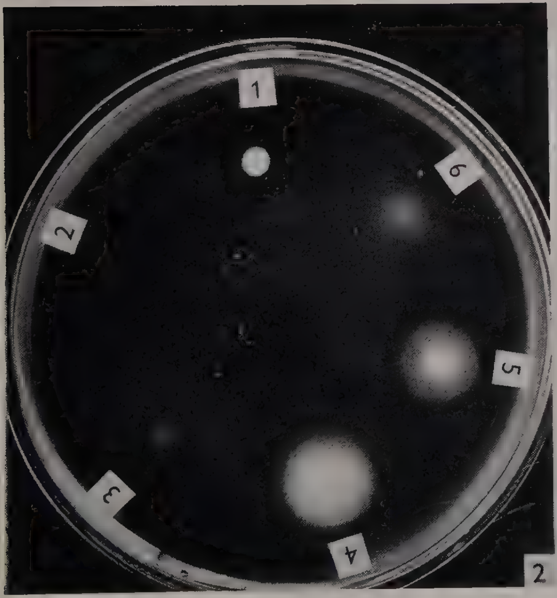

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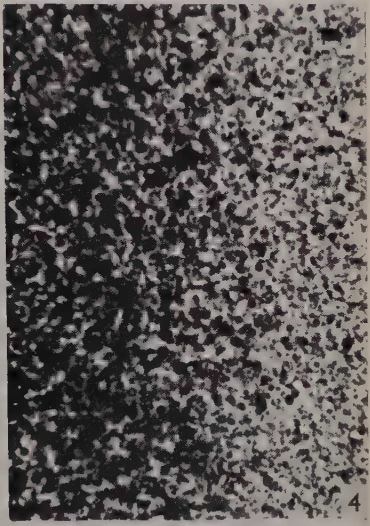

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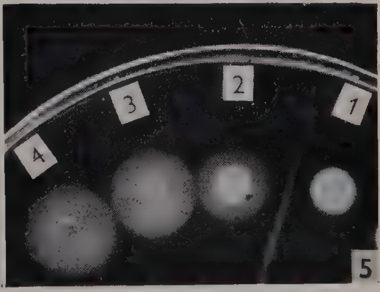

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G. Miller & M. Aschner—Auxanographs with yeasts. Plate 1

KNOX, R. & COLLARD, P. (1952). *J. gen. Microbiol.* 6, 369–373.

The Effect of Temperature on the Sensitivity of *Bacillus cereus* to Penicillin

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SUMMARY: A strain of *Bacillus cereus* known to produce penicillinase adaptively was tested for penicillin sensitivity at different temperatures. With small inocula, the organisms grew freely at 37° in concentrations of penicillin up to a hundred times greater than those which completely suppressed their growth at 42°. This difference in apparent sensitivity almost disappeared when the inoculum was very heavy or consisted of cells which had been allowed to adapt by previous growth in the presence of penicillin.

The penicillinase of *Bacillus cereus*, NRRL 569 was shown by Pollock (1950) to be formed adaptively. He also found that 'no significant amount of enzyme was formed at 18°, and about four times as much at 35° as at 30°'. In earlier work on the adaptive formation of bacterial tetrathionase (Knox & Pollock, 1944), it had been found that the process of adaptation was much more sensitive to heat than was the fully formed enzyme (Pollock, 1945), while the known growth-promoting effect of tetrathionate in semi-anaerobic conditions (Knox, 1945) was suppressed at temperatures such as 42° at which adaptive formation of the enzyme was also suppressed (Knox, 1950).

In view of these facts, it was decided to study the effect of temperature on the growth of a known penicillinase-producing organism in the presence of penicillin.

METHODS

The organism used was *B. cereus*, NRRL 569 obtained from Dr Pollock. Cultures were maintained by serial subculture at weekly intervals on slopes of nutrient agar kept at room temperature. From these, 6 or 18 hr. broth cultures were used to inoculate experimental tubes or flasks. In most experiments 6 in. × $\frac{5}{8}$ in. tubes were incubated in water-baths having a range about the stated temperature of $\pm 0{\cdot}5°$. In a few experiments 250 ml. conical flasks were used to obtain better aeration by increasing the surface/volume ratio. Growth after 18 hr. incubation was measured roughly by visible opacity (recorded as + or 0), or, where quantitative comparisons were wanted, on the density scale (at λ 400) of a Unicam spectrophotometer; rectangular cells 1 cm. in depth were used, with a sample of uninoculated medium as a 'blank'. It was found that with an 18 hr. culture in broth a density reading of 0·2 was given by 285×10^6 cells/ml. (dry weight 0·27 mg./ml.).

RESULTS

The effect of temperature on growth in nutrient broth

In nutrient broth in the absence of penicillin it was found that the organisms grew well over the range 22–45° but failed to grow at 48°. At 45° quantitative measurements of growth could not be made because of pellicle formation.

Table 1 shows the results of inoculating 0·02 ml. of an 18 hr. broth culture into 10 ml. of nutrient broth incubated for 18 hr. at different temperatures.

It is clear that the organisms grew adequately up to temperatures as high as 42°, though, as would be expected, growth in flasks was much heavier than in tubes. In another experiment good growth was also obtained up to 42° in tubes of broth inoculated with 0·02 ml. of a 1/1000 dilution of an 18 hr. broth culture.

Table 1. *Effect of temperature on growth of* B. cereus *in broth*

	Temperature			
	22°	37°	42°	45°
	Opacity (spectrophotometer readings)			
6 in. × ⅝ in. tubes	0·165	0·125	0·115	P+
250 ml. flasks	0·540	1·000	0·620	P+

P+ = pellicle growth. Inoculum: 0·02 ml. 18 hr. broth culture in 10 ml. medium. Incubation: 18 hr.

Table 2. *Effect of temperature on growth of* B. cereus *in penicillin broth*

	Temperature			
	22°	37°	42°	45°
	Opacity (spectrophotometer readings)			
1/1 inoculum	0·085	0·195	0·18	0
1/1000 inoculum	0·015	0·2	0	0

Inoculum: 0·02 ml. 18 hr. broth culture. Incubation: 18 hr. in 6 in. × ⅝ in. tubes.

The effect of temperature and inoculum size on growth in penicillin broth

In broth containing 10 units of penicillin/ml. good growth was obtained up to 42° when a large inoculum was used, but only up to 37° when the inoculum was a thousand times smaller (Table 2).

The effect of different inocula upon the penicillin sensitivity at 37 and 42° was then further investigated. 0·02 ml. of 1/1, 1/10, 1/100 and 1/1000 dilutions of a 6 hr. broth culture of *B. cereus* were inoculated into tubes of broth containing 0, 1, 10, 100 and 1000 units of penicillin/ml. (Table 3A). It is evident that inoculum size was much more critical at 42° than at 37°.

The effect of previous adaptation on growth of different inocula in penicillin broth at 37° and 42°

Pollock (1950) showed that maximal adaptive production of penicillinase demanded conditions of full aeration. In the experiment shown in Table 3, three sets of inocula were prepared from cultures of *B. cereus* grown in broth for 6 hr. at 37°—A in a 250 ml. conical flask, B in a 6 in. × ⅝ in. tube containing 1 unit of penicillin/ml. broth and C in a 250 ml. conical flask containing 1 unit of penicillin/ml. broth. Each inoculum was standardized to the same opacity and 0·02 ml. of different dilutions inoculated into tubes of broth as shown. After 18 hr. incubation the following results were obtained: (1) the level of penicillin in which the organism grew was in all cases a function of inoculum size; (2) at 37° there was no difference between the levels tolerated by equal inocula of the three types of cell; (3) at 42° the adapted cells were able to grow in from 10 to 100 times greater concentrations of penicillin than the unadapted cells; (4) with the smaller inocula the cells adapted in the flask grew in even higher concentrations than those adapted in the tube.

Table 3. *Effect of inoculum size and previous adaptation on growth in penicillin broth at 37 and 42°*

Inoculum		Temperature									
		37°					42°				
		Penicillin concentration (units/ml.)					Penicillin concentration (units/ml.)				
Cells grown 6 hr. at 37° in 10 ml. broth in	Dilution in- oculated	0	1	10	100	1000	0	1	10	100	1000
A 250 ml. flask without penicillin	1/1	+	+	+	+	+	+	+	+	±(+)	0(+)
	1/10	+	+	+	+	0	+	±(+)	0(+)	0	0
	1/100	+	+	+	0	0	+	±(+)	0	0	0
	1/1000	+	+	+	0	0	+	0(+)	0	0	0
B 6 in. × $\frac{5}{8}$ in. tube with penicillin 1 unit/ml.	1/1	+	+	+	+	+	+	+	+	+	+
	1/10	+	+	+	+	0(+)	+	+	+	0(+)	0
	1/100	+	+	+	0(+)	0	+	+	0	0	0
	1/1000	+	+	+	0	0	+	+	0	0	0
C 250 ml. flask with penicillin 1 unit/ml.	1/1	+	+	+	+	+	+	+	+	+	+
	1/10	+	+	+	+	0(+)	+	+	+	+	0(+)
	1/100	+	+	+	0	0	+	+	+	0	0
	1/1000	+	+	+	0	0	+	+	0	0	0

+ = good growth; ± = faint growth; 0 = no growth. All tubes were examined after incubating for 18 hr. and then for a further 72 hr. Brackets indicate those tubes which showed a change, e.g. 0(+) = no growth at 18hr., good growth after a further 72 hr.

It should be noted that when the tubes were incubated for a further 72 hr., growth was found to have occurred in many of the cultures which had previously shown no growth, though others showed no growth after still further incubation, even in the presence of added penicillinase (Glaxo Laboratories, Greenford).

DISCUSSION

It seems that the apparent tube sensitivity of an organism is the resultant of a number of factors of which inoculum size and temperature are perhaps the most obvious. The effect of inoculum size upon the apparent sensitivity of penicillinase-producing organisms has been known for some years and has been used by Luria (1946) as the basis of a test for differentiating such organisms from other penicillin-resistant strains. The possible effect of temperature has not been so widely appreciated.

The most reasonable explanation of our findings appears to be as follows: All cells of *B. cereus* 569 possess a basal quantity of penicillinase which can hydrolyse penicillin at 37 and 42°. The *adaptive production* of penicillinase can take place at 37° but not at 42°. Growth cannot take place at penicillin concentrations above a certain level, approximately 1–2 units/ml. The fate of cells inoculated into penicillin containing media depends partly on the initial penicillin level and partly on the available penicillinase, which is itself determined by the size of the inoculum and the possibility of adaptation. There will therefore be a longer or shorter lag period during which penicillin is being destroyed but no growth is possible until this falls below the critical level. If sufficient cells survive this period, then growth will occur on prolonged incubation; on the other hand, the cells may suffer irreversible damage and die.

The experiments here described and the results obtained previously with tetrathionase provide two clear examples of the importance of adaptive mechanisms *in bacterial growth.* Both tetrathionase and penicillinase can be formed by a process of enzymic adaptation in conditions which exclude natural selection. Both adaptive processes are more sensitive to heat than the process of growth in the organisms investigated. In both cases it can be shown that if conditions are suitably chosen adaptation is a necessary condition for growth, and when penicillin is the substrate, it may even be a necessary condition for survival, since sensitive cells will be destroyed by penicillin if they cannot produce enough penicillinase to neutralize it first.

Adaptive mechanisms of this kind may be of vital importance for bacterial growth not only in the laboratory, but also in the animal body. For example, one way in which pyrexia could benefit the host is by suppressing adaptive mechanisms essential for survival and multiplication of the parasite. If this were so, reduction of body temperature by the use of antipyretic drugs could even swing the balance in favour of the parasite. Pasteur and his colleagues observed in 1878 (Pasteur, Joubert & Chamberland, 1878) that fowls naturally immune to anthrax could be infected if the body temperature were artificially reduced. Pasteur's conclusion that the high body temperature (42°) of the fowl suppressed the growth of *B. anthracis* was criticized when it was found that the organism grew well in the laboratory at 42–43°. But this criticism would lose its force if the virulence of *B. anthracis* were found to depend on a thermolabile adaptive mechanism of the kind we have here described.

Artificially induced pyrexia, local or general, has been used in the treatment of a number of infections and some benefit might be expected from combining it with the use of antibiotics. This could occur in two ways: (1) as suggested by Marks (1951), in a recent paper on the effect of temperature on the sensitivity of tubercle bacilli to antibacterial agents, by suppressing the growth of all organisms except a few rare variants resistant to both the antibiotic and the higher temperature; (2) by the suppression of adaptive mechanisms in the parasite as, for example, in mixed infections complicated by penicillinase-producing organisms.

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The Homofermentative Lactobacilli of Apple Juice

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SUMMARY: Three species of homofermentative lactobacilli, identified as *Lactobacillus delbrueckii*, *L. plantarum* and *L. leichmannii* have been found to be of widespread occurrence on apples and in the expressed juice. Precautions must be taken to arrest their development during the processing of apples intended for the production of unfermented apple juice, otherwise the juice will be rendered unpalatable through development of excess acidity.

Some of the bacterial disorders of cider described by Warcollier (1928), such as Graisse disease and mannite disease, are caused by lactobacilli of the heterofermentative type which produce not only lactic acid but also acetic acid, alcohols and carbon dioxide from carbohydrates. An excellent account of the occurrence and development of these organisms in cider has been published by Steuart (1949).

Homofermentative lactobacilli producing mainly lactic acid and only traces of by-products from carbohydrates are also present in apple juice. They occur in large numbers on apples and we have observed considerable fluctuations in their population during growth and maturity of the fruit on the tree. Freshly expressed juice from sound fruit always contains a small number of these bacteria, but when the fruit contains a large proportion of rot their incidence in the juice increases. For the production of pure natural apple juice, the juice is always fined and filtered prior to bulk storage in refrigerated pressure tanks, and this treatment decreases the complement of organisms in the juice. No multiplication occurs during bulk storage of carbonated juice at a temperature of 37°, in fact, the organisms tend to diminish with prolongation of the storage period. Since no account of the presence of homofermentative lactobacilli in apple juice appears to have been published we describe in this paper three organisms of this group which we have encountered on fruit and in the juice during the production of pure natural apple juice at a fruit juice factory in Gloucestershire.

METHODS

Bergey's Manual (1948) describes eleven well defined species of homofermentative lactobacilli, and divides them into two subgenera based upon their optimum growth temperatures. We have used this key for identifying our isolates. As a group, these organisms occur as long, slender rods; they are Gram-positive, catalase negative, micro-aerophilic, non-spore forming, and convert carbohydrates into lactic acid with small amounts of by-products.

We used Blickfeldt's solution agar for isolation and both this medium and apple-juice agar for observation of morphological and colony characteristics. Blickfeldt's solution and apple juice were used for examination of growth in liquid media. Biochemical reactions were carried out on 2 % (w/v) solutions of the appropriate substrate in yeast water.

ORGANISMS ISOLATED

Many cultures of homofermentative lactobacilli were encountered and were eventually sorted into three distinct types. These were allotted culture numbers and are described below.

Culture R. 31. Isolated from fresh juice and from apples used for the production of juice, was present on sound fruit and also on fruit infected with Blue Mould (*Penicillium expansum*) and with Brown Rot (*Sclerotinia fructigena*), thus indicating a wide distribution. It was also encountered in tank-stored juice but rapidly diminished during storage. On nutrient agar at 25 and 30° it appeared as non-sporing, non-motile, single rods 6–7 × 1 μ., and also in short chains. The rods were Gram-positive and catalase negative. After 7–14 days some curved forms were observed. On apple-juice agar, colonies appeared in 2 days, attaining a diameter of 2 mm. after 3 days. Colonies were refractile, mucoid, slimy and moist, with edges entire, round, and raised centre. In liquid media a uniform turbidity was produced at 25 and at 30°; after 4 days, a deposit was produced, with very little surface growth, and the organism appeared to be a faculative anaerobe. Optimum growth developed within the range 40–45°. Lactic acid was produced from glucose, dextrin, sucrose and maltose, but not from lactose.

These characters correspond to those of *Lactobacillus delbrueckii* (Leichmann) Beijerinck.

Culture W. 19. This organism was present on apples and in the expressed juice at all stages of processing, but rapidly decreased during bulk storage. On apple-juice agar, it appeared as a short rod 2 × 0·5–1 μ. with a tendency to barrel and coccoid form. It was Gram-positive, catalase negative, non-spore forming, and non-motile. Colonies appeared very similar to those of Culture R. 31. In liquid media a slight surface growth appeared in 8 days, assuming the form of an extremely thin surface film, which when disturbed, broke into small fragments and sank. Growth in apple juice was slow with very little surface growth, a faint turbidity appearing after 4 days accompanied by a slight deposit. The organism was microaerophilic with an optimum temperature of 30–37° and produced lactic acid from lactose, sucrose, maltose, glucose and mannose.

Its identity was established as *L. plantarum* (Orla-Jensen) Holland.

Culture R. 34. This was encountered on apples and in the juice at all stages of processing. On apple-juice agar, it occurred as short slightly curved rods 1·5 × 0·5 μ. In old cultures there was a tendency towards a coccoid appearance with slight chain formation. The rods were Gram-positive, catalase negative, non-motile and did not produce spores. Colony appearance was similar to that of the two previous species. The organism was microaerophilic with optimum growth at 30° and in liquid media formed a deposit and only a slight film after 7 days. Acid was produced from lactose.

The above characteristics established its identity as *L. leichmannii* Bergey *et al.*

DISCUSSION

L. plantarum and *L. leichmannii* are found widely distributed in nature in fermenting plant and animal products, and *L. delbrueckii* is invariably found in fermenting vegetable and grain mashes.

We have found all three organisms to be of frequent occurrence in apple juice, and although they are less harmful there than organisms of the heterofermentative group it is desirable that adequate precautions be taken to arrest their development before they produce excess acidity and render the juice unpalatable. Fortunately their growth is retarded in juice which is carbonated and stored in bulk at low temperatures. Their inactivation in juice pasteurized in bottle has been made the subject of a separate study.

We wish to thank the Directors of Seager, Evans and Co., for permission to publish this paper.

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On the Presence of *Acetobacter oxydans* in Apple Juice

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SUMMARY: *Acetobacter oxydans* has been identified in pure natural apple juice. A new technique which possesses some advantages over existing methods has been developed for demonstrating capsule formation by this organism and by other species of *Acetobacter*.

Grove (1915) isolated five different types of acetic acid bacteria from thirty to forty ciders and described their cultural characters when grown in malt extract solution containing 2 % ethanol; the most commonly occurring was *Bacterium xylinum* but the others were not identified. Vandecaveye (1927) reported two strains of vinegar bacteria which did not form a pellicle, observed during an examination of cider vinegar. These organisms produced large quantities of acetic acid in fermented ciders and were catalase positive. Using their oxidative powers as a basis of differentiation between the species, he identified his strains as *Acetobacter aceticum* and *A. rancens*.

During the course of a microbiological investigation carried out at a commercial apple juice factory in Gloucestershire, we isolated and identified a large number of yeasts, moulds, and bacteria present on the fruit that were capable of inducing spoilage in apple juice. Their ability to develop in apple juice and their resistance to pasteurization were observed and will be reported elsewhere. Among the bacteria encountered were two species of *Acetobacter*, of which one was identified as *A. xylinum* Brown, similar to *Bact. xylinum* found in cider by Grove. The other species appeared to be a variety of *A. oxydans* (Henneberg) Bergey *et al.* Since the presence of this latter organism in apple juice has not hitherto been recorded we describe its characters in the present paper.

It was first isolated from a mixed plate culture which had been seeded with a sample of apple juice taken after several months' bulk storage at 37° of a heavily carbonated juice. The incidence of the organism increased from the third to the tenth month of storage to a maximum of 900 organisms/ml. juice. Thereafter, a decrease was observed, and 6 months later the organism was detected with difficulty. High carbonation and low temperature sufficiently arrested its development and prevented any impairment of the organoleptic properties of the juice. Subsequent investigations showed that it was frequently present on the apples used for processing and its presence in the juice was ascribed to this source.

METHODS

Bergey's Manual (1948) has reclassified the entire group of vinegar bacteria under the genus *Acetobacter*, all species possessing the common characteristic of forming at some period of their development either a pellicle or a surface film on the surface of suitable liquid media. It describes seven principal species, together with two species considered by Beijerinck to be varieties of *A. rancens*, and also seventeen ancillary species which it is declared require further study before any change in nomenclature is advisable.

As a group the acetic acid bacteria are all Gram-negative, non-spore forming, and give a negative response to the Voges-Proskauer reaction. They oxidize ethanol to acetic acid, and various organic compounds to organic acids. Walker & Tošić (1943) have stated that, with the exception of *A. peroxydans*, all known *Acetobacter* species are catalase positive. Kulka & Walker (1946) established that capsule formation also is probably a characteristic feature of the genus. The keys to the identification of certain species given by *Bergey's Manual* (1948), Shimwell (1948), and Walker & Tošić (1946*a*, *b*), were found helpful for identifying our isolates.

Media. Apple juice and Blickfeldt's solution were used as liquid media. These solutions were used for preparation of solid media with 2 % agar, but for this purpose apple juice was usually diluted to decrease the sugar content to 1–2 % and 0·2 % w/v $(NH_4)_2HPO4$ added.

Gram reaction. The reliability of the staining technique was checked by mixing together on the slide containing the unknown organism a Gram-positive organism (*Micrococcus candidus*) and a Gram-negative organism (*Bacterium coli*).

Catalase test, was carried out according to the method used by Walker & Tošić (1943) using sterile malt extract (sp.gr. 1036) buffered with $CaCO_3$ and solidified with 1·5 % agar.

Biochemical reactions were conducted in yeast water with addition of 1% (w/v) of the appropriate sugar or organic substrate. The tests were incubated at 25° and the tubes examined at intervals up to 2 weeks.

Capsule staining. To a 5 % (w/v) solution of gelatin in water at 60° sufficient Cotton Blue powder is added to give an intense dark blue colour forming the contrast medium, powdered eosin (1–2%) is then added, and the whole thoroughly mixed. Films are prepared on a warm stage at 30–37°. A little of the liquid gelatin stain is placed on a wet film on a warm slide of the organism under study. Observation is carried out on the warm stage after mixing and covering with a warm cover-glass. The bacterial cells appear pink against a blue background, the capsule remaining colourless. Thin films are easily prepared by this method. To assist differentiation and to prevent coalescence of capsules, the culture may previously be diluted with very dilute sodium hydroxide. This method does not involve drying or undue manipulation of the material and damage and distortion to the capsule are minimized. Observations of the capsules of both *A. xylinum* and *A. oxydans* by this method revealed a characteristically uniform appearance although slight differences in measurement, as compared with Indian Ink methods, were sometimes detected.

RESULTS

Cultures were obtained from apples and from juice. In the former case epidermal swabs were made and teased out and suspended in sterile water. After making suitable dilutions these were plated on suitable solid media.

When examining juice for bacteria we found it convenient to centrifuge the samples and to wash the deposit with distilled water before making a film, as this avoided the juice charring or becoming a sticky mass.

The following morphological and cultural characters and biochemical reactions were found.

Solid media. On apple-juice agar and Blickfeldt's solution agar at 25° the organism appeared barrel-shaped, with a tendency to be coccoid. After 24 hr. the colony appeared clear, colourless and circular with a mucoid tendency. After 48 hr. it appeared greyish and opaque and the mucoid tendency had increased. After 60 hr. it became grey-white, circular, refractile, entire edge, mucoid in texture, the centre uniform and slightly globose. After 70 hr. the colony was 2·5 mm. in diameter, round, globose, grey-white in colour and opaque, thickly mucoid, entire edge, raised and less refractile than at 48 hr.

Slope cultures. At 24 hr. on apple-juice agar slopes, the colony was similar in appearance to that on agar plates. After 72 hr. the colony had begun to run along the surface towards the bottom of the slope. This was observed at 20° and at 25°.

Apple-juice agar stab. Stab cultures at 25° showed very slight growth along the line of inoculation for a distance of 5–6 mm. Good growth developed on the surface. After 3 weeks the growth was more luxuriant than that obtained by any other cultural method. After 6 weeks the surface had become stiff in texture. The colony was still viable up to 12 weeks at 20° and 25°.

Gelatin media. No liquefaction in 3 weeks.

Liquid media. Under anaerobic conditions there was no growth in apple juice. Under aerobic conditions at 25° the organism exhibited formation of diplococci with a tendency to short chain formation. After 48 hr. a slight surface scum appeared as a greyish white haze, which became uniform through the medium by 72 hr. and ultimately disappeared. After 7 days a thin greyish white film had spread over the entire surface, with a slight tendency to climb the walls of the glass. This film sank when disturbed.

Optimum temperature. The organism grew well at 20–25°, between 25° and 30° growth was less good, and above 30° restriction became even more pronounced.

Motility. Neither cultures in sloppy gelatin nor liquid medium showed motile organisms.

Gas formation. Gas formation was not evident in aerobic culture in glucose-yeast water, ethanol-yeast water or acetic acid-yeast water at 25°.

Biochemical reactions. Acid was produced from glucose and sucrose accompanied by heavy growth, and from maltose, lactose, galactose, and dextrin with slight growth. There was very little growth and only a slight acid formation in the presence of raffinose. Acid was produced in the presence of 2 % ethanol accompanied by fair growth.

Other characters. The organism was Gram-negative, catalase positive, non-spore forming, and gave a negative response to the Voges-Proskauer reaction. The presence of a capsule was demonstrated, by our own technique and by the methods of Gin and Baker. The following measurements were obtained:

Gin's method: $1{\cdot}2 \times 1\mu$. to $1{\cdot}4 \times 1{\cdot}2\mu$.
Baker's method: $1{\cdot}2 \times 1{\cdot}2\mu$. to $1{\cdot}4 \times 1{\cdot}5\mu$.
Our method: $1{\cdot}3 \times 1{\cdot}3\mu$. to $1{\cdot}5 \times 1{\cdot}5\mu$.

DISCUSSION

Presence of a capsule, negative responses to Gram staining and the Voges-Proskauer reaction, absence of spore formation, production of catalase, together with ability to produce acetic acid from sugars and ethanol establish the organism as a species of *Acetobacter*. Non-oxidation of acetic acid, absence of pigment formation, and optimum growth at 20–25° eliminates all the principal species in *Bergey's Manual* with the exception of *A. oxydans*. *Bergey's Manual* mentions, however, that motile forms of the latter organism occur and that the organism has been encountered in the form of rods, $0{\cdot}8$–$1{\cdot}2 \times 2{\cdot}4$–$2{\cdot}7\mu$., either singly or in chains. Another difference from our own organism is that according to *Bergey's Manual*, *A. oxydans* does not produce acid from lactose.

These differences may be reconciled by study of other publications. It is possible that our organism was cultivated at too high a temperature for motility to be observed, for Jorgensen (1939) remarked that *Bact. oxydans* [syn. *A. oxydans* (Henneberg) Bergey *et al.*] can be induced to produce motile cells by cultivation at 20° on wort agar followed by transplantation into diluted beer. Shimwell (1948) confirmed that in many species of acetobacter motility sometimes occurs only at certain stages in the development of the culture. He also stated that although all members of the genus are rods, in some the cells are so short and so ellipsoidal and even coccoid in form that they are frequently mistaken for true cocci. Jorgensen also asserted that the cells of *Bact. oxydans* may appear in pairs or as fairly long chains.

The ability of our organism to produce acid from lactose and from other sugars is a characteristic shared by *A. industrium* (Henneberg), Bergey *et al.* Walker & Tošić (1946*b*), however, claimed that *A. oxydans* can also produce acid from lactose, but Shimwell regarded *A. oxydans* and *A. industrium* as identical. Jorgensen makes a distinction between the two species for he remarked that although both organisms form a thin film on liquid media, that of *A. oxydans* tends to climb the glass (a characteristic exhibited by our organism), whereas that formed by *A. industrium* falls to the bottom. On these grounds, therefore, we feel justified in identifying our organism as *A. oxydans*.

We wish to thank the Directors of Seager, Evans and Co., for permission to publish this paper.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Thirteenth General Meeting at Manchester University on Tuesday and Wednesday, 18 *and* 19 *September* 1951. *The following communications were made:*

COMMUNICATIONS

A SYMPOSIUM ON THE RHIZOSPHERE

Chairman: H. G. Thornton (*Rothamsted Experimental Station, Harpenden*)

General Introduction. By P. W. Brian (*I.C.I. Butterwick Research Laboratories, Welwyn*)

The Rhizosphere in Relation to Plant diseases. By S. D. Garrett (*School of Botany, Cambridge University*)

Mycorrhiza. By J. L. Harley (*Botany Department, Oxford University*)

Root-nodules of Leguminous Plants. By P. S. Nutman (*Rothamsted Experimental Station, Harpenden*)

ORIGINAL PAPERS

Observations on the Morphology and Natural Relationships of *Rhizobium*.

By K. A. Bisset (*Department of Bacteriology, University of Birmingham*)

The formation of spores by *Rhizobium* has many times been reported but is still not generally accepted. In the course of numerous isolations from root-nodules of a large variety of species of Leguminosae several strains of sporogenous *Rhizobium* have been isolated. These strains were often weakly Gram-positive in young cultures but soon adopted the typical, small, Gram-negative morphology of the genus. Selection of strains capable of surviving boiling produced some which were strongly Gram-positive in young cultures and lost this character very gradually, as the culture aged.

Cultures of strains from lupin and lucerne, which had survived boiling, were inoculated in seedlings of the appropriate species of plant, and produced nodules under controlled conditions, verifying their identity.

The Gram-positive *Bacillus*-like bacteria differed from true *Bacillus* in several particulars, having the *Rhizobium* characters of producing coccal swarmers with a single, polar flagellum, and of adopting the form of branched 'bacteroids', not only in root-nodules, but also in culture medium of acid pH, which several species of *Bacillus* failed to do when examined. The mode of spore formation was slightly different. They also differed in producing gas in carbohydrates, when these were fermented, which is very rare in *Bacillus* except *B. polymyxa*. Fermentation reactions were alike in the Gram-positive, forms and in the typical, *Rhizobium*-like forms from any one source; a further confirmation of their identity, as these reactions were very variable. Some fermented no carbohydrates at all.

It appears probable that *Rhizobium* is a highly specialized member of the Bacillaceae, related to the *B. polymyxa* group, which exhibits the same tendency to revert to the appearance of a Gram-negative bacterium.

The Vitamin Requirements of Lactic Acid Bacteria Isolated from Beer and Brewers' Yeasts.

By Conrad Russell (*College of Technology, Manchester*)

The vitamin requirements of ten species of lactic acid bacteria found in beer and brewers' yeasts have been investigated. Three of these species were new ones, and, in all, thirty-four strains were studied. The medium and technique were essentially that of Dunn, Shankman, Camien & Block (1947), except that the organisms were incubated in an atmosphere of 80–85 % CO_2, 20–5 % air, and growth was estimated turbidimetrically.

The organisms included strains of the following species: *L. pastorianus, L. buchneri, L. bifidus, L. leichmanni, L. plantarum, S. damnosus* and *S. cremoris*. The number of organisms requiring the stated vitamins are shown in parenthesis: pantothenic acid (33), nicotinic acid (23), riboflavin (4), aneurin (2), *p*-aminobenzoic acid (1), biotin, choline, inositol, folic acid and the B_6 complex were not required by any of the organisms.

The number of vitamins required by any one organism varied from none to four, as follows (number of requirements shown in parenthesis): one strain (0), eleven strains (1), sixteen strains (2), five strains (3), one strain (4).

The strains of any given species did not show homogeneity in their requirements, although strains of the same species isolated from the same sample of beer or yeast tended to be more homogeneous than those of the same species isolated from different sources.

The absence of any requirements for biotin or folic acid is particularly to be noted, as is the lack of *any* vitamin requirements by one of the organisms.

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Cultural Studies with Holotrich Ciliates of the Sheep's Rumen.

By Brenda Sugden and A. E. Oxford (*Aberdeen*)

Although the holotrichs inhabiting the sheep's rumen constitute only a minor portion of the total ciliate population, they are worth study because of the speed with which they convert soluble sugar into their own granular storage starch. Some limited success has been achieved in their anaerobic cultivation *in vitro* in a simple salt medium, changed daily, which was based on that used by Hungate for rumen oligotrich ciliates. It contained in addition a suitable sugar, usually cellobiose (0·1 %), and a source of organic nitrogen provided by cleared and Seitz-filtered rumen liquor (10 %). Survival, after initial multiplication, was 8 days or more as against 2–3 days, without multiplication, in presence of non-utilizable, or no, carbohydrate. Suitable soluble sugars included galactose, raffinose and salicin as well as those previously mentioned by Masson and Oxford as leading to rapid storage-starch production.

The sugar plus rumen liquor could be replaced with advantage (particularly as regards longevity) by considerably less boiled and filtered grass juice, but not red clover juice. Although bacteria alone could not serve as a complete food, as shown by the failure of a maltose or pentose medium, supporting a dense bacterial population, to prolong protozoan life, yet no successful method of cultivation from well washed single cells has been devised. Various antibiotics and bacterial inhibitors were found to be harmful to the protozoa also.

The rapid rate of conversion of sugar to storage starch was confirmed microscopically by the appearance of iodophilic granules in starved cells within 5 min. of adding the sugar. *Isotricha*, but not *Dasytricha*, could utilize small vegetable (e.g. rice) starch grains swallowed whole but starch as paste was not utilized.

Microbiological Synthesis of Fat. Some Effects of Vitamins and Amino-acids on Fat Production by Moulds.

By MALCOLM WOODBINE (*Division of Industrial Biochemistry, Faculty of Technology, Manchester* 1)

Of forty-two potential fat-producing moulds (Woodbine, Gregory & Walker, 1951), *Penicillium spinulosum*, *Aspergillus nidulans* and *P. javanicum* have been examined for their response to vitamins and amino-acids. Results exhibited a graduation of effect in that *A. nidulans* was somewhat inhibited by both aneurin and the 'omnibus', based on that of Cutts & Rainbow (1950); *P. javanicum* was favourably affected to a slight extent by aneurin (1 μg./ml.), and *P. spinulosum* was unaffected by the presence of either.

Previous workers have shown that amino-acids stimulate yeast. Garton, Goodwin & Lijinsky (1951) have found leucine, glycine, valine and alanine best for lipid production in *Phycomyces Blakesleeanus*. First experiments with *Penicillium spinulosum* showed that, of the twenty-two amino-acids, valine and NH_4NO_3 (1:1 on N content) gave enhanced fat production. The work was extended to all three moulds, using glycine, alanine, tyrosine, methionine and valine. Results with *A. nidulans* clearly demonstrated the superiority of NH_4NO_3 alone in terms of percentage and coefficient of fat; that no nitrogen source, other than NH_4NO_3, held any particular advantage for *P. javanicum* and that valine was used as easily as NH_4NO_3 by *P. spinulosum*.

(This work was initiated by the Chemical Microbiology Committee of the M.R.C. and the author was seconded from Scientific Adviser's Division, Ministry of Food, to enable him to undertake this work with Dr T. K. Walker.)

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Microbiological Synthesis of Fat. Effects of Varying Ionic Concentrations on Fat-producing Moulds. By José Garrido and Malcolm Woodbine (*Division of Industrial Biochemistry, Faculty of Technology, Manchester* 1.)

Previous work (Gregory, 1950; Woodbine, Gregory & Walker, 1951; Woodbine, 1951) has shown that *Penicillium spinulosum*, *Aspergillus nidulans* and *P. javanicum* are potential fat producers, and Steinberg (1945) has demonstrated the minimum ionic concentration/maximum growth concept with moulds. Using a basic medium of Prill, Wenck & Peterson (1935) and incubating for 8 days at 25°, the optimum concentrations of magnesium sulphate (25–500 mg. %), phosphorus, as sodium dihydrogen phosphate (6·5–73·1 mg. %), and potassium, as potassium sulphate (10·8–21·7 mg. %) have now been obtained for these three moulds. Using these results, eighteen media were prepared which enabled the reaction of the moulds to varying concentrations of one ion in presence of constant concentrations of the other two ions to be determined. *A. nidulans* is easily the best fat-producer (50 % fat on dry felt; fat coefficient 17; economic coefficient 34) but the *Penicillium* spp. are of interest because of their concomitant production of citric acid (Birkinshaw & Raistrick, 1931; Lockwood, Ward, May, Herrick & O'Neill, 1934).

This work was initiated by the Committee on Chemical Microbiology of the M.R.C. and carried out under the direction of Dr T. K. Walker. One of us (M.W.) was seconded from the Scientific Civil Service and one (J.G.) was a Juan de la Cierva Foundation (Madrid) Fellow.

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The Effect of Enzyme Inhibitors on the Multiplication of Coliphage T_2. By J. H. Czekalowski (*Department of Bacteriology, The School of Medicine, Leeds*)

The Behaviour of an Iridescent Lysogenic Strain of *Pseudomonas pyocyanea*. By Loïs Dickinson (*Research Department, Boots Pure Drug Co. Ltd. Nottingham*)

Two compounds, which inhibited the action of several bacteriophages of *Ps. pyocyanea* on an indicator strain of this organism, inhibited iridescence in all the cultures tested. Surface active agents dispersed iridescence but did not suppress phage action. One iridescent lysogenic culture showed two definite steps in phage increase. One occurred at 1½–2 hr. (the same latent period as when free phage acted on an indicator host) and another at 4–5 hr. The first of these was inhibited by the compounds but the second was not, and the final phage titre of unfiltered cultures was not significantly different from the control culture. Unfiltered 24 hr. cultures of the iridescent strain had a viable colony count of about 10^8/ml., whereas the phage count, as assayed against the indicator strain in an 18 hr. assay, was only about 10^6/ml. If the assay plates were incubated for a further 30 hr. after the 18 hr. reading, then more plaques developed and the phage titre rose to about 10^8/ml. Sterile filtrates of the culture behaved similarly but when these filtrates were grown on the indicator strain only 18 hr. type plaques were obtained. Another iridescent lysogenic strain and a non-iridescent lysogenic strain behaved similarly.

Iridescence was not apparent on a chemically-defined medium solidified with agar; neither was phage lysis on such medium clear-cut. There is no conclusive evidence that iridescence must necessarily be dissociated from phage activity.

An Organism of the Pleuropneumonia Group Needing for Growth a Factor in Mucin.

By D. G. ff. Edward and W. A. Fitzgerald (*Wellcome Research Laboratories, Beckenham, Kent, and Wellcome Veterinary Research Station, Frant, Sussex*)

In isolating P strains of pleuropneumonia-like organisms from the genital tract of cattle it was often noted that colonies only appeared under flakes of vaginal exudate included in the inoculum; such strains were difficult or impossible to subculture (Edward, 1950). Good growth could be obtained by subculture on a plate spread with a drop of a 2 % suspension of hog gastric mucin, when there was no growth without mucin. By the use of mucin pleuropneumonia-like organisms were isolated from a specimen of semen, which was negative when cultured on the ordinary medium. Mucin promoted the growth of all except one of twenty freshly isolated P strains. The degree to which it was needed varied; some strains grew without mucin, although growth was improved by it. Adaptation quickly took place so that after about five subcultures all strains grew as well without mucin.

Growth was not promoted by other viscous substances, such as gum acacia and 'Manucol'. A purified preparation of the polysaccharide fraction of hog gastric mucin was inactive, but a fraction containing the factor concerned in enhancing bacterial virulence (Smith, 1951*a*) was equally as active as crude mucin in promoting growth. Human saliva, bovine nasal mucus and bovine vaginal mucus from an animal not in oestrus also promoted growth; human saliva also contains the virulence enhancing factor (Smith, 1951*b*). It is possible that the factor promoting growth is the same as that enhancing virulence. Alternatively promotion of growth by Smith's preparation of the virulence enhancing factor may be due to an impurity, as it has not yet been obtained as a homogeneous material.

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Some Aspects of the Growth and Metabolism of *Proteus vulgaris* with Limiting Concentrations of Nicotinic Acid.

By F. L. Jackson (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The growth of a strain of *Proteus vulgaris* in a simple synthetic medium, containing lactate as sole carbon source and limiting concentrations of nicotinic acid, has been studied under controlled conditions. Dry weight and cell nitrogen estimations indicate that, after the disappearance of detectable quantities of nicotinic acid from the medium, a phase of approximately linear protoplasmic synthesis occurs before the cessation of growth. The curve of total bacterial numbers as estimated by direct counts follows a different course; and remains practically exponential during the phase of approximately linear synthesis. This difference is due to reduction in size of the organisms.

The oxygen uptake of samples of culture becomes linear during the phase of linear synthesis. After this, there is a gradual falling off of oxygen uptake. addition of more nicotinic acid to the culture is followed by a prompt increase in oxygen uptake.

From the time at which nicotinic acid is no longer detectable, pyruvate accumulates in the medium. This accumulation continues after the cessation of growth and the quantity present may exceed 3 mg. pyruvic acid/ml. No pyruvate accumulates in cultures containing an excess of nicotinic acid. The addition of more nicotinic acid to a culture which is accumulating pyruvate is followed by a rapid decrease in the pyruvate level. Aneurin and cocarboxylase are very much less effective than nicotinic acid in promoting the disappearance of pyruvate. The addition of aneurin to the medium before incubation does not prevent the accumulation of pyruvate.

Induced Variations in a Penicillin-Resistant Staphylococcus with special Reference to Reduction of the Resistance.

By A. Voureka (*Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London*)

Bacterial variants were obtained by exposing staphylococcal cells to substances toxic to the organism. The strain chosen was mouse virulent, coagulase positive, phage typable, and resistant to ten units of streptomycin and 400 units of penicillin. Cells derived from a single organism were used, to have a more uniform population, and the experiments were made on a small number of cells so that all could be examined.

Experimental

To part of a suspension of young bacterial cells an equal amount of broth containing chloramphenicol (40–125μg./ml.) was added. After 6–24 hr. incubation, 0·025 ml. drops of the mixture were spread over the length of a blood agar plate and incubated.

As a control, part of the same suspension was similarly diluted and plated, but before incubation. (By observing incubated cells exposed to the drug it was ascertained that division was not occurring. Occasionally there were one or two divisions.) Whilst all control colonies were identical with the parent strain, a number of the colonies yielded by the treated bacteria showed gross reduction of resistance to penicillin and to streptomycin. They were also mouse avirulent, coagulase negative, phage untypable, grew poorly, and when first isolated consisted largely of swollen cocci.

After fifty subcultures some characters remained unchanged; in some variants there was partial reversion of resistance to penicillin. One only slightly altered variant reverted completely to normal.

It is believed that the variations appeared following damage to the parent cell and that selection of natural mutants can be excluded.

The experiments were successfully repeated with penicillin, terramycin, nitrogen mustard and hydrogen peroxide.

Special attention was given to penicillin because of the paradox of recovering penicillin sensitive bacteria after exposing penicillin resistant ones to high concentrations of penicillin for a sublethal time.

Experiments, *in vivo*, using penicillin and terramycin gave similar results.

Genetic Analysis without Sexual Reproduction by Means of Polyploidy in *Aspergillus nidulans*. BY G. PONTECORVO AND J. A. ROPER (*Department of Genetics, The University, Glasgow*)

It is now possible (Roper, *Experientia*, in the press) to produce strains carrying in their hyphae and in their uninucleate conidia diploid nuclei heterozygous for known genetic 'markers', and in their ascus primordia tetraploid nuclei. Through *sexual reproduction* (ascospores) these diploids give origin to the haploid and polyploid types expected in the case of tetraploid inheritance; through *vegetative reproduction* (hyphae or uninucleate conidia) they remain indefinitely diploid, but yield occasionally diploids homozygous for one or more of the 'markers'.

Five linked genes (w, ad_1, ad_2, y and bi, in this sequence) and two on different chromosomes (lys and orn_1) located by means of standard genetic analysis, have been used to analyse the types of homozygous diploids produced vegetatively by four different strains of diploids, each heterozygous for four or more of these genes. It works out that diploids homozygous for one of the linked markers are usually homozygous also for all the other linked markers *either* on its right *or* on its left on the chromosome map, but not for those on the other side or on different chromosomes. Thus the homozygotes seem to arise through some sort of 'mitotic' or 'somatic' crossing over, similar to meiotic crossing over but not followed by reduction. If these deductions were right, the centromere would be located between ad_1 and ad_2, which are more than 20 units apart on the genetic map. An independent localization by means of ascus dissection is in progress.

This type of genetic analysis, not making use of sexual reproduction is applicable to other moulds, yeasts and bacteria. It permits to identify linkage groups and sequences of genes irrespective of the existence of a sexual cycle.

A Serological Variant of a Staphylococcal Typing Bacteriophage. By Joan E. Rippon (*Staphylococcal Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9*)

The staphylococcal typing bacteriophage 42D, as first isolated, was of serological group B_2. It has now been shown that several batches contain a phage of serological group F. Such batches of phage show very little change in their lytic action from the B_2 phage. All the phages of serological group F previously isolated lyse an entirely different set of strains.

Tests have shown that some of the earlier batches of apparently pure B_2 type 42D phage contain a very small quantity of the F type. This phage has increased in proportion in subsequent propagations until in our latest batch of phage it has completely superseded the B_2 serological type.

By propagating from single plaques, picked after neutralization of an apparently pure B_2 phage with an anti-serum prepared against a B_2 phage, it has been found that variation from the B_2 to F 42D phage may take place although the reverse change has not yet been demonstrated.

DEMONSTRATIONS

Genetic analysis without Sexual Reproduction by means of Induced Polyploidy in *Aspergillus nidulans*. By G. Pontecorvo and J. A. Roper (*Department of Genetics, Glasgow University*)

The Behaviour of an Iridescent Lysogenic Strain of *Ps. pyocyanea*. By Loïs Dickinson (*Research Department, Boots Pure Drug Co. Ltd., Nottingham*)

Microbiological Synthesis of Fat. A Stainless Steel Pilot-scale Fermenter of the Modified-Waldhof Type. By José Garrido and Malcolm Woodbine (*College of Technology, Manchester*)

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